Universidade de Aveiro Departamento de Biologia
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FABIANNE DE ARAÚJO RIBEIRO

O FLUXO DE NANOPARTÍCULAS DE PRATA NUMA CADEIA TRÓFICA AQUÁTICA

SILVER NANOPARTICLES FLOW IN AN AQUATIC TROPHIC CHAIN

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Susana Patrícia Mendes Loureiro, Investigadora auxiliar do Departamento de Biologia e CESAM, Universidade de Aveiro.

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palavras-chave

Nanopartículas de prata, prata iónica, *Daphnia magna*, *Pseudokirchneriella subcapitata*, *Danio rerio*, *Carassius auratus*, bioacumulação, transferência trófica

resumo

As nanopartículas de prata (AgNP) têm sido produzidas e utilizadas em uma grande variedade de bens de consumo, desde produtos de higiene pessoal a embalagens de alimento e utensílios médicos. A ação antimicrobiana das nanopartículas de prata é o principal fator que as torna úteis e ideais para tais aplicações. A prata é um metal não essencial e pode ser encontrado no ambiente em concentrações ecologicamente irrelevantes. No passado, a atividade de revelação fotográfica era a principal fonte de prata no ambiente. Ultimamente, estas concentrações têm aumentado devido à vasta utilização das nanopartículas de prata na indústria. A presença da prata no ambiente pode constituir um risco para as espécies e os efeitos causados podem ser do tipo letal ou sub-letal. Para além disso, a exposição dos organismos à prata, mesmo que não os leve à morte imediata, pode causar uma acumulação deste metal, e que poderá ser transferido entre os níveis tróficos da cadeia alimentar aquática. Tendo isto em consideração, o objetivo deste trabalho foi estudar a transferência das nanopartículas de prata numa cadeia trófica aquática modelo, e comparar os mesmos processos com a exposição a nitrato de prata ($AgNO_3$). Para alcançar este objetivo, este trabalho foi dividido em quatro estudos: avaliação da toxicidade das AgNP e do $AgNO_3$ para as espécies em estudo, uma posterior avaliação da bioconcentração das AgNP e do $AgNO_3$ pela alga verde *P. subcapitata,* o estudo da bioacumulação da prata em *Daphnia magna*, exposta a diferentes vias de contaminação (água, alimento e ambos) e por último a avaliação da transferência das AgNP e de $AgNO_3$ através de um desenho experimental que incluiu o peixe *Carassius auratus* expostos a água e alimento contaminados. Os resultados obtidos com estes estudos indicam que a bioacumulaçãoo da prata na alga *P. subcapitata* ocorre devido à internalização dos iões de prata, e não das nanopartículas. Estas aparentemente encontram-se em aglomerados próximas às células das algas, não entrando nas células/algas. Relativamente à *Daphnia magna*, o maior fator de bioacumulação foi obtido quando estas foram expostas à água e alimento contaminados com AgNP. Finalmente observou-se que os peixes não atingiram um equilíbrio na concentração interna de prata, e que o órgão que apresentou maior bioacumulação de prata foi o fígado. Para além disso, foi verificada no fígado uma taxa de eliminação muito baixa, o que nos pode levar a sugerir que as nanopartículas de prata podem persistir neste órgão. Apesar de não se verificar um potencial para transferência trófica, as nanopartículas de prata podem representar risco para as espécies aquáticas aqui estudadas.

keywords

Silver nanoparticles, ionic silver, *Daphnia magna*, *Pseudokirchneriella subcapitata*, *Danio rerio*, *Carassius auratus*, bioaccumulation, trophic transfer

abstract

Silver nanoparticles (AgNP) have been produced and applied in a variety of products ranging from personal care products to food package containers, clothing and medicine utilities. The antimicrobial function of AgNP makes it very useful to be applied for such purposes. Silver (Ag) is a non-essential metal for organisms, and it has been historically present in the environment at low concentrations. Those concentrations of silver increased in the last century due to the use of Ag in the photographic industry and lately are expected to increase due to the use of AgNPs in consumer products. The presence of AgNP in the aquatic environment may pose a risk for aquatic species, and the effects can vary from lethal to sublethal effects. Moreover, the contact of aquatic organisms with AgNP may not cause immediately the death of individuals but it can be accumulated inside the animals and consequently transferred within the food chain. Considering this, the objective of this work was to study the transfer of silver nanoparticles in comparison to silver ions, which was used as silver nitrate, within an aquatic food chain model. To achieve this goal, this study was divided into four steps: the toxicity assessment of AgNP and $AgNO_3$ to aquatic test-species, the bioaccumulation assessment of AgNP and $AgNO_3$ by *Pseudokirchneriella subcapitata* and *Daphnia magna* under different exposure scenarios, and finally the evaluation of the trophic transfer of Ag through an experimental design that included the goldfish *Carassius auratus* in a model trophic chain in which all the species were exposed to the worse-case scenario. We observed that the bioconcentration of Ag by *P. subcapitata* is mainly driven by ionic silver, and that algae cannot internalize these AgNPs, but it does internalizes dissolved Ag. *Daphnia magna* was exposed to AgNP and $AgNO_3$ through different exposure routes: water, food and both water and food. The worse-case scenario for Daphnia Ag bioaccumulation was by the joint exposure of contaminated water and food, showing that Ag body burdens were higher for AgNPs than for $AgNO_3$. Finally, by exposing *C. auratus* for 10 days through contaminated water and food (supplied as *D. magna*), with another 7 days of depuration phase, it was concluded that the 10 days of exposure were not enough for fish to reach a plateau on Ag internal concentration, and neither the 7 days of elimination were sufficient to cause total depuration of the accumulated Ag. Moreover, a higher concentration of Ag was found in the intestine of fish when compared with other organs, and the elimination rate constant of AgNP in the intestine was very low. Although a potential for trophic transfer of AgNP cannot be suggested based in the data acquired in this study, there is still a potential environmental risk for aquatic species.

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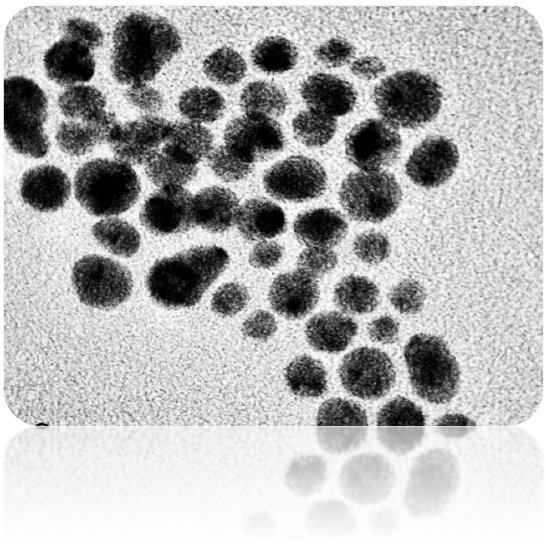
Chapter 1

General Introduction



1 GENERAL INTRODUCTION

1.1 Background & Scope

A nanomaterial is, by definition, a "*natural, incidental or manufactured material containing particles in an unbounded state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range of 1nm-100nm"* (EU, 2011a). Particles on this size-range and with a specific surface area by volume greater than 60 m^2/cm^3 (EU, 2011a), that constitute a nanomaterial, are defined as nanoparticles (NP´s). Their presence in our everyday life has become a reality, and the actual observed tendency is that the incorporation of particles into consumer goods will increase with time. Due to their small size, NP´s possess intrinsic properties that make them of great interest for industry. The application and use of NP´s containing products will ultimately lead to the entrance of nanoparticles and/or their transformation products into the environment. The release of toxic substances into the natural environment is usually regulated by local or general authorities through laws that are established by considering the risk that a certain substance may represent to the environment. For nanoparticles, such a regulation is under construction because of their relatively new increased production and application. When building such regulations for nanoparticles there are several aspects that should be considered, given that NP´s may not behave as regular contaminants when it

comes to their interactions with biotic and abiotic components. Several studies are already available on NP´s effects on aquatic (freshwater and marine) species from different trophic levels as well for terrestrial species. Those studies help regulatory bodies to build the laws for rational and safe use of nanoparticles.

The ongoing discussion within the European legislation is whether nanoparticles should be embraced in the existing law for regular chemicals such as REACH, or else, if there should be an implementation of a new document to regulate nanoparticles use and waste treatment, as well as their allowable environmental concentration. Additionally, one should consider that apart from REACH, there are specific regulations in Europe concerning the use of biocides and pesticides, and given the broad use of nanomaterials, the same nanoparticle may fill the requirements to be regulated by more than one legislation.

Metal-based nanoparticles are nowadays the most used NP´s on the market, and among metal-based NP´s, silver nanoparticles (AgNP) are the most "popular" and therefore, applied in a greater variety of products than any other metal-based nanoparticle. Given that, the concentration of Ag originated from AgNP is expected to increase in the environment, potentially increasing the risk for aquatic and terrestrial species. Because silver can be considered the most toxic metal that has been already found in the environment even at very low concentrations, and due to the potential increase of environmental silver concentrations related to the use of AgNP, this thesis will deal with the biological aspects guiding interactions of AgNP with three different freshwater species that constitute a model trophic chain. This study aimed at investigating if there are

differences in the toxicity and bioaccumulation of AgNP compared to ionic Ag, by the three model species, representing three different levels of an aquatic trophic chain. Moreover, if the potential of trophic transfer is higher for nanoparticles compared to ionic silver. In order to answer those questions, this work adopted a step-by-step approach in which exposures were carried out in single or combined trophic levels. The experimental method will be described in detail at the end of this chapter, and additional information regarding the general subject of this thesis is given now forward.

1.2 Silver Nanoparticles

Silver nanoparticles (AgNP) differ from macro forms of silver in many properties, such as size, distribution and morphology. The most important among them is that the smaller size of particles provides a larger ratio of surface area to volume (>60 m^2/cm^3) (EU, 2011a). This characteristic can increase the potential for silver ions to be released from silver nanoparticles, as well as the possibility for AgNP to enter into biological compartments when compared to bigger particles (Purcell & Peters, 1998; Sotiriou & Pratsinis, 2010). AgNP are usually manufactured as a colloidal suspension, in which nanoparticles have a surface charge that can attract atoms with an opposite charge, creating a double layer of atoms on the surface of nanoparticles. This double layer "travels" with the nanoparticle as it flows throughout the suspension. The electrical potential at the boundary of this double layer is known as the Zeta potential of the particles and has values that typically range from +100 mV to -100 mV (B. J. Kirby &

Hasselbrink, 2004; D. L. Liao, Wu, & Liao, 2009; Panáček et al., 2006; Sotiriou & Pratsinis, 2010). Usually nanoparticle suspensions with a zeta potential higher than +25mV or lower than -25mV are known to have good stability.

Silver nanoparticles are produced by the reduction of silver salts ($AgNO_3$) through the application of reducing agents into the reaction system. Reducing agents can be either from chemical or biological nature. Among the chemical reducing agents citrate and saccharides such as glucose and maltose (B. J. Kirby & Hasselbrink, 2004; D. L. Liao et al., 2009; Panáček et al., 2006) are commonly used, while for biological reducing agents, plant leaf extracts (Song & Kim, 2008), microorganisms (Klaus, Joerger, Olsson, & Granqvist, 1999), and enzymes can be applied in the production of silver nanoparticles. During manufacturing process of AgNP, the size and coating of resulting particles can be controlled by monitoring the chemical and physical properties of the reaction, such as temperature, light and chemical composition of the capping agent (Zhang et al., 2003). Surface coating provides the functionalization of particles considering the objectives that the particle was designed for. For instance, silver nanoparticles designed for antimicrobial purposes have different surface functionalization than silver nanoparticles design for the treatment of human cancer cells (Ahamed, AlSalhi, & Siddiqui, 2010); or even AgNP manufactured to be applied in fabrics (socks, clothes, bed lines, etc) are expected to have a coating material that will allow its persistence in the product for a longer period of time (S. Silver, Phung, & Silver, 2006).

1.3 Characterization of AgNP

The ecotoxicological assessment of nanoparticles in the environment requires a fully characterization in order to understand the behavior and consequently possible interactions that nanoparticle will have with the organisms and surrounding media. Therefore, a number of techniques are available to characterize nanoparticles both qualitatively and quantitatively. In this thesis, we will focus on the characterization methods that are relevant for environmental studies.

Dynamic light scattering (DLS) is used to determine the dynamic size distribution pattern of colloidal suspensions of nanoparticles, through time variation of light scattered from suspended particles that are under Brownian motion. The basic principle of DLS is that small particles in solution, when heated by a light with a wavelength greater than the size particles, disperse the light to every direction, which provides the observation of a time-dependent fluctuation in the scattering intensity.

Electrophoretic light scattering (ELS) is based on the same principle of DLS, with the difference that DLS measurements are based on the mobility of particles whereas ELS measurements relies on the oscillating electric field present in the suspension. ELS is used to measure zeta potential of nanoparticles in solution. Zeta potential, unlike particle size or molecular weight, is a property involving not only the particles but also their environment characteristics, e.g., pH and ionic strength (S. Silver et al., 2006; Xu, 2008).

Electron microscopy (EM) techniques allow the characterization of particles regarding their original size and shape, with high magnification efficiency.

Scanning electron microscopy (SEM) is based on the interaction of an electron beam with the particle surface, resulting in a three-dimension image. In addition to the surface characteristics and topography of the particles, SEM can also provide information on its chemical composition by X-ray microanalysis, which is resulted by the interaction with the electron beam with the sample. *Transmission Electron Microscopy* (TEM) provides greater magnification for the analysis of particle size and shape but only in a two-dimension outlook. On TEM analysis, a beam of electrons is projected (in vacuum) onto a very thin section of the sample, and electrons interact with the sample as they pass through, to form an image that is magnified and focused by an image device coupled in the TEM. In addition to the much greater resolving power of TEM, other valuable information for nanoparticles characterization can be obtained using electrons as illumination source, such as: chemical composition, by the use of *Energy Dispersive X-Ray Spectroscopy* (EDXS), oxidation state, by using *Electron Energy Loss Spectroscopy* (EELS) and crystal structure, from *selected area and convergent beam diffraction* (SADP, CBED). Sample preparation for both SEM and TEM represents a crucial step in EM analysis, and it is sometimes challenging for the characterization of nanoparticles under relevant ecotoxicological test-conditions, due to media chemical composition. Another obstacle faced by the use of EM for NP characterization is concentration. In order to achieve an effusively information on particles features, samples should contain a high concentration of nanoparticles, which is usually far from a relevant concentration applied in the ecotoxicity assessments.

Field-Flow-Fractionation (FFF) is a useful technique to characterize NP under environmental matrices. FFF is based on a flow-assisted hydrodynamic

separation that permits the physical separation of small quantities of macromolecules and particles. The FFF mechanism works by a field that is applied on the NP suspension pumped through a narrow tube or channel perpendicular to the direction of flow, in order to induce the separation of particles present in suspension, which is caused by their different mobility response to the force exerted by the field (Kammer, Legros, Hofmann, Larsen, & Loeschner, 2011; Xu, 2008). With the advance of nanotechnology and the increasing number of studies regarding the effects of nanoparticles in the environment, the characterization techniques of nanoparticles are constantly being improved in order to meet the needs of a fully understanding of NPs behavior under laboratory test-conditions.

1.4 Silver Nanoparticles in The Environment

Before going into detail on the presence of silver nanoparticles and their fate in the environment, one should know that silver (Ag) is a naturally occurring element in the earth´s crust, present at low concentrations. However at some locations in the world silver is present in rather higher concentrations, which allows its extraction through mining activities (Kammer et al., 2011; Purcell & Peters, 1998). The first human-derived presence of Ag in the environment came from mining areas and from the use of silver in the photographic industry and water purification in swimming pools (Seltenrich, 2013).

The most abundant forms of silver present in the environment are silver sulfides (AgS_2) and silver chloride complexes ($AgCl_n^{1-n}$). In a typical oxygenated

freshwater environment, the fraction of Ag free ion (Ag^+) is often 40% of the total dissolved silver concentration (See Cornelis et al (2010) and chapter 3 for definition on dissolved metal), but usually this concentration is considerably lower, depending on the percentage of dissolved organic carbon (DOC) in water, to which Ag^+ can bind, thus decreasing the free ion concentration (Hogstrand & Wood, 2009). In summary, under freshwater conditions, the fate of silver will be governed by the concentration of chlorides, sulfates and DOC in the water (Hogstrand & Wood, 2009). Those components are likely to modify silver toxicity by inducing its chemical speciation.

In addition to what is above mentioned and discussed about the sources and the behavior of silver in the environment, silver nanoparticles represent an extra source of Ag to nature. However, AgNP will reach the environment through a variety of process occurring since the manufacturing of nanoparticles, the incorporation into products, the transport of Ag-containing products, and ultimately through the use of these Ag-containing products. For instance, a study on the effluent composition of a nano-silver washing machine proved that silver nanoparticles were found in the effluent in an average concentration of 10.9 (±7.1) μg L^{-1}(Farkas et al., 2011). Similar to the AgNP washing machine, many products containing AgNP are now available on the market for consumption. A project on emerging nanotechnologies (pen) has set an online database that lists all products available on the market that contains nanoparticles. The search for silver nanoparticles products reveals the occurrence of 395 products on the database (http://www.nanotechproject.og), last visited in December 2013. Among these products we can find sheet sets, socks, towels, kitchenware, pillows, face creams and throat sprays. These were cited here because they are

more likely to release AgNP into the surrounding media (air or water) either through washing or direct use. Considering that the effluent and the release rate of nanoparticles from all these products are not characterized, we can expect nothing but the presence of AgNP or related Ag forms in different environmental compartments, with unknown consequences for wildlife and human health. Once they reach the environment, AgNP may undergo different process that will ultimately change their initial characteristics. As a result they can become either less or more toxic, depending on their bioavailability to the species in each environment. Considering the aquatic compartment as a model, figure 1.1 demonstrates a brief and ideal diagram of the behavior of silver nanoparticles once they have reached a freshwater environment. If AgNP will reach the aquatic compartment as single particles, they tend to form agglomerates and/or aggregates (figure 1.1, process 1). Usually in the literature the discrimination between agglomerates and aggregates is often confused or mixed up. Particles forming agglomerates are attracted by van der Waals' forces and can return to their original state, i.e., single particles, which make agglomeration a reversible process. Instead, aggregation is a non-reversible process, i.e., particles will remain as aggregates through their life cycle (Baalousha, 2009). Both process are extremely dependent on the ionic strength of the media, and consequently the zeta potential of the suspension (B. J. Kirby & Hasselbrink, 2004).

The stability of a colloidal suspension can be explained by the DLVO theory. According to this theory, the forces between surfaces or colloidal particles can be considered as the sum of two different forces: the short-range, attractive van der Waals forces and the long-range, repulsive electrical double-layer forces. The

interaction between these two forces has consequences on the colloid stability (Kammer et al., 2011; Liang, Hilal, Langston, & Starov, 2007)

A colloidal suspension with low ionic strength will be constituted of particles with high surface charge, thicker electrical double layer and will be governed by dominant repulsive forces between particles, agglomeration will be lower. On the contrary, a media with high ionic strength will induce a thinner double layer around the particles, lower surface charges, and which will have a greater tendency of particles to form agglomerates (Jiang, Oberdörster, & Biswas, 2008; Purcell & Peters, 1998).

Additionally, the aggregation process can be classified into two types: heteroaggregation and homoaggregation. If a colloidal suspension is composed by monodispersed similar particles, they will form homoaggregates whilst if a colloidal suspension is formed by dissimilar particles then heteroaggregation is likely to take place (Islam, Chowdhry, & Snowden, 1995). As a result of agglomeration/aggregation, particles will form large clusters that, being formed by smaller clusters with different density, mass distribution and porosity they will be subject to the entrance of the solvent phase of the colloid, resulting in sedimentation, which is schematized in the figure 1.1 as process 4.

Following agglomeration/aggregation, dissolution is another mechanism that nanoparticles are subject to experience under environmental conditions, especially in oxidizing circumstances. Through dissolution silver ions can be released from the surface of nanoparticles (Misra, Dybowska, Berhanu, Luoma, & Valsami-Jones, 2012), and become reactive with other constituents of the surrounding media, which will lead to the formation of silver complexes (figure 1.1, process 3). Dissolution is schematized in figure 1.1 as process 2.

The mechanism happens through a cooperation process involving protons and dissolved oxygen. In a solution containing no other oxidants or reducing agents, the global equation for dissolution should be:

$$2\,Ag_{(s)} + \tfrac{1}{2}\,O_{2(aq)} + 2H^{+}_{(aq)} \longleftrightarrow 2Ag^{+}_{(aq)} + H_2O_{(l)}$$

According to a study performed by (Liu, Sonshine, Shervani, & Hurt, 2010), the oxidation of AgNP is likely to take place through a simple redox reaction that produces hydrogen peroxide (H_2O_2) as an intermediate. In the same study, the formation of H_2O_2 was not detected under dissolution of $AgClO_4$, which indicates that H_2O_2 production is intrinsic to AgNP dissolution. Environmental factors such as pH, temperature and the presence of natural organic matter (NOM) can influence dissolution (Li, Lenhart, & Walker, 2010). In addition, lower pH´s will induce higher dissolution rates (Ho, Yau, Lok, So, & Che, 2010).

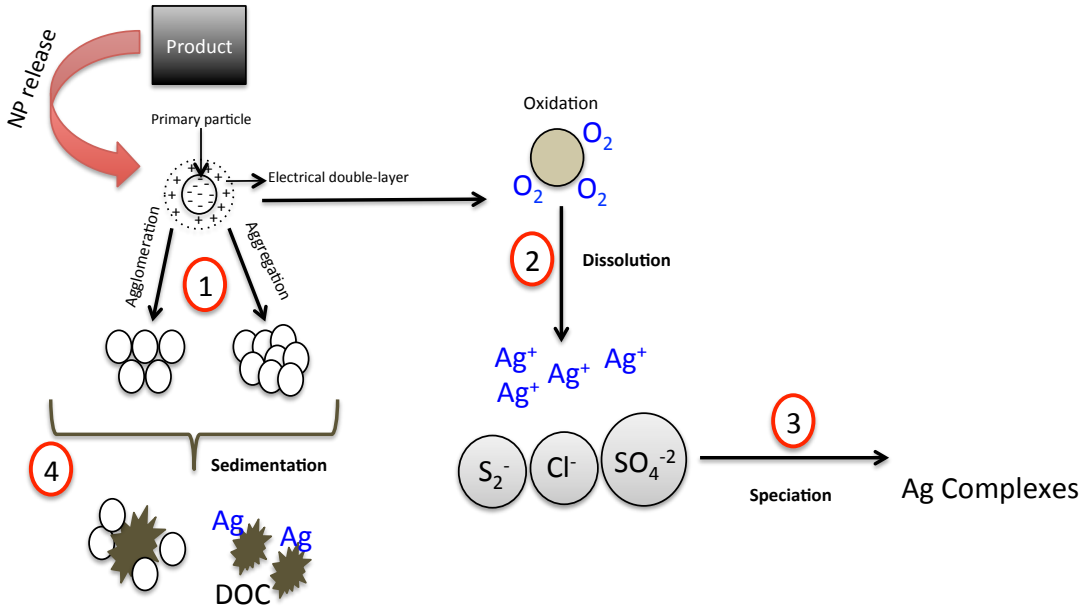


Figure 1.1. Schematic representation of silver nanoparticles (AgNP) release from the product and the consequent behavior of nanoparticles in freshwater environments.

1.5 Ecotoxicity and Bioaccumulation

Animals living in a certain environment are constantly interacting with their surrounding media. Some of these interactions lead to the uptake of xenobiotic substances introduced in their habitat by human activity. Ecotoxicity is the research field that studies the negative effects that a toxicant exposure may cause on a single organism and also be transposed to the population level (Escher & Hermens, 2004). The effect of a toxicant in aquatic organisms is usually reported on the basis of its external concentration, i.e., the dose that was applied in a test-system. For instance, in order to predict the risk that a silver enriched effluent from a fabric will cause to a zooplanktonic species (e.g. *Daphnia magna*), several concentrations of silver will be applied in the test-

media containing a number of organisms, and the LC_{50} (Lethal concentration that affects 50% of the exposed animals) will be reported based on the concentration of silver in the water (e.g. µg/L) (see OECD, 2004 for reference on *Daphnia* sp. acute immobilisation test). However, not all the silver that was primarily dosed in the test-system will be available to cause toxicity on Daphnia. Similarly to other metals, silver can react with other components of the media, forming chemical complexes (figure 1.1, process 3) that could not be bioavailable for uptake by Daphnia. Unlike metals, organic compounds tend to be less bioavailable with time because of their degradation rate or volatility in the water (Boudina, Emmelin, Baaliouamer, Grenier-Loustalot, & Chovelon, 2003). As the need to understand bioavailability of chemicals was evolving, a few models were proposed with the effort of improving risk assessments. The FIAM (Free ion activity model) was first proposed in the 1980s and attempt to link metal uptake from solution with dissolved metal speciation in the environment (Campbel, 1995). Later on, the BLM (Biotic ligand model) essentially with the same basis as the FIAM, takes a further step in attempting to directly link metal speciation in the water with uptake, bioaccumulation and toxicity (Di Toro et al., 2001; Paquin et al., 2002).

Bioavailability refers to the fraction of the toxicant that is freely available to the organism (Van Leeuwen et al., 2005). One example to understand bioavailability is to think of a polar bear in the North Pole that is thirsty but he only had frozen freshwater in the form of icecaps. The water may be there but is not available.

Just like icecaps, chemicals can be present in the environment but in a immobilized state which is not bioavailable to the organisms and unlike to exert toxicity. For this reason, the assessment of a toxic effect should attempt to cover

the steps leading from nominal to free concentrations, from free to internal concentrations, and ultimately from internal to target concentrations (Escher & Hermens, 2004).

Regarding internal concentration of a toxicant in an organism, it can happen as a result from the uptake of free (or bioavailable) fraction of the chemical in the environment. This uptake is possible to occur via different routes, which are covered by the bioaccumulation concept.

The term bioaccumulation refers to the accumulation of a substance inside the organism, which can happen either via the surface of the body or via food ingestion. Therefore, a few definitions are used to distinguish bioaccumulation through distinctive routes. *Bioconcentration* is used to describe the uptake of a substance through the surrounding media (e.g. Figure 1.2 A). The mechanism of uptake varies among species and their habitat. *Bioaccumulation* is applied to describe the uptake of a substance via both the surrounding media and through dietary exposures, as exemplified in the figure 1.2 by the process B. Finally *Biomagnification* is related to the concentration of a substance in the organism, taken restrictedly from its (contaminated) diet. Biomagnification can only be assumed to occur if the Bioaccumulation Factor (BAF) in higher trophic levels (e.g., fig 1.2, D) is higher than the BAF from lower levels in the trophic chain (e.g. Fig 1.2 C).

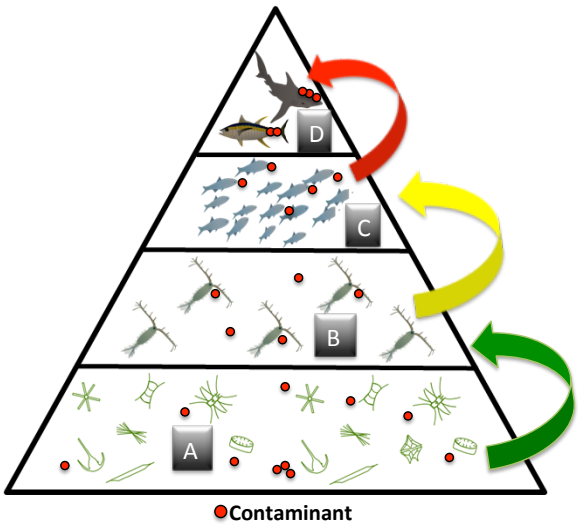


Figure 1.2 Representation of an aquatic trophic chain and the flow of contaminants from the baseline producers to the final consumers. Bioconcentration happens on the basis of the trophic chain (A), in which microalgae uptakes chemicals from the water. On the next levels (B, C, D) both bioaccumulation and biomagnification are likely to occur. Biomagnification will happen if the Bioaccumulation Factor (BAF) value from level D is higher than the BAF value from level C.

The mechanism of bioaccumulation of a compound in an organism is subject to several environmental variables such as the concentration of the substance in the environment, bioavailability and exposure, and also to physiological variables such as uptake route, kinetics and biotransformation of the substance. Understanding bioaccumulation requires the assessment of the mechanisms ultimately leading to the internal concentration of a compound inside the organism. Those mechanisms can be divided into three dynamic processes: the chemical speciation of a substance, specially in the case of metals, which depends on the pH, hardness and organic matter content of the media, followed by the assimilation and elimination rates of the substance by the organism (toxicokinetics) (figure 1.3) and finally by the internal translocation of the

chemical, in which it can be storage either as a non-toxic form, or it can exert toxicity in the target organs (toxicodynamics) (Escher et al., 2010).

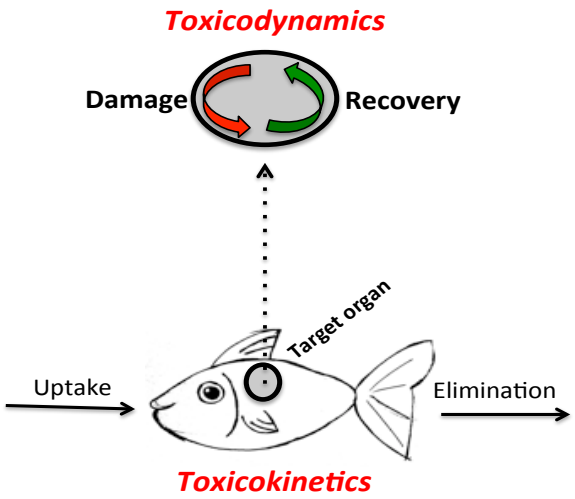


Figure 1.3. Different processes involved in the mechanism of internal accumulation of a chemical. *Toxicokinetics* is related to the uptake and elimination constants, while toxicodynamics is related to the effects of internal concentration, such as damage of a target organ/tissue, metabolism, detoxification, excretion and damage recovery.

In this thesis, a toxicokinetic model was applied to describe uptake and elimination of silver, as silver nanoparticles (AgNP), dissolved silver compounds (Ag_{dis}), and ionic silver (Ag^{+}) using aquatic model species. The model aims at describing the internal fate of silver by using a one compartment model and explain body burden by the balance between uptake and excretion processes. Through the quantitative aspects of the model, it can be expected that the internal concentration of a substance in the organism is a result of the uptake and elimination constants and the external concentration of the substance. Therefore, assuming that exposure concentration remains constant, the model equation for *uptake* should be:

$$Q(t) = \frac{k1}{k2} \times C_{exp} \times (1 - e^{(-K2*t)}) \qquad \textbf{Equation 1}: \textit{Uptake}$$

Where:

Q(t) is the concentration in the organism, at time t (e.g µg/g dry weight)

k1 is the uptake rate constant (L/g/hour)

k2 is the elimination rate constant (1/hour)

C_{exp} is the exposure concentration (e.g. µg/L)

e is the exponential function

Equilibrium is reached when the concentration in the organism reaches a plateau, i.e., when internal concentration no longer increases with time and uptake and excretion are balanced. Under this circumstance, the Bioconcentration Factor (BCF) may be calculated as:

$$BCF = \frac{k1}{k2}$$

Or

$$BCF = \frac{C_{organism}}{C_{exp}}$$

Where $C_{organism}$ is the concentration in the organism (µg/g dry weight) and C_{exp} is the exposure concentration (µg/L).

When the exposure period stops and organisms are changed to clean media, an exponential decrease on body concentration is expected to occur. The decrease on body concentration is dependent on the elimination rate constant and the internal concentration of the body. Therefore, the mathematical expression for elimination is:

$$Q(t) = \frac{\mathrm{k1}}{\mathrm{k2}} \times C_{\mathrm{exp}} \times (1 - e^{(-\mathrm{k2} * (\mathrm{t} - \mathrm{tc}))} - e^{(-k2 \times t)})) \quad \textbf{Equation 2}: \textit{Elimination}$$

Where tc is the time in which animals are transferred to a "clean" exposure media, i.e., free from the contaminant. The toxicokinetics half-life is the time needed to eliminate half of the initial amount of the substance that was accumulated. The elimination rate constant (k_2) is inversely proportional to the half-life: a substance with a longer half-life will induce a lower elimination rate. For instance, lipophilic compounds are known to have higher half-life, thus inducing low elimination rates. That is one of the reasons why bioconcentration of organic substances is related to the lipid content of the organism. Furthermore, assuming that uptake remains constant, an increased k_2 will lead to slower elimination, longer time needed to reach equilibrium and higher BCF values.

1.6 Model Species

Raphidocelis subcapitata (Hindak, 1990)

Raphidocelis subcapitata is a freshwater microalgae species with a relatively wide distribution in the rivers and water bodies around Europe and Africa (Aruoja, 2011). Algae cells in culture are generally solitaire, except during cell division, where small clusters can be seen inside colorless mucilage. The cells have a helical shape (figure 1.4, left) and range in size from approximately 5µm to 11µm. As a general characteristic of the phylum Clorophyta, *R. subcapitata* is a eukaryotic cell with chloroplasts containing chlorophyll pigments, which are

responsible for their green color. Reproduction happens mostly sexually with alternations of generations (Leliaert et al., 2012).

Microalgae such as *Raphidocelis subcapitata* are in the basis of freshwater trophic chains and are responsible for approximately half of the global primary production and atmospheric oxygen. This circumstance places algae in a very important ecological position, which makes them a species of interest in ecological risk assessment. Being the basis of aquatic trophic chains implies that any damage or harmful effect caused to algae population will be reflected on the higher trophic levels of the aquatic food chains. Likewise, as the energy in the system flows from the primary producers to its primary and secondary consumers, similar mechanisms may be responsible for the trophic transfer of toxic substances first accumulated by algae.

Figure 1.4. Electron microscope (Raman-CARS) image of *Raphidocelis subcapitata* cells (left); algae cells suspended in test-media of silver nanoparticles in a laboratory set up experiment (right).

Daphnia magna (Straus, 1820)

The genus *Daphnia* is one of the most widely known groups of freshwater invertebrates. *Daphnia* is most diverse and abundant in temperate regions, but it has representation through all climate zones and continents, and is one of the dominant members of the world's freshwater zooplankton. *Daphnia* reproduces largely by cyclic parthenogenesis: females lay eggs into the brood pouch that do not require fertilization, and which produces only females; after many cycles of this asexual process, and usually in response to adverse environmental conditions (e.g., temperature, population density, pH, etc), the parthenogenesis females produce males or mixed brood of males and females. Some evidences suggests that the induction of sexual females can be due to changes in photoperiod, food levels or crowding while males can be induced by photoperiod or by a chemical sign emitted when there is a high density of females (Ferrari and Hebert 1982). Sexual females produce haploid eggs, which are fertilized by males and develop later in diapausing embryos, encased in a protective structure called ephippium. These resting eggs can hatch when the environmental conditions become favorable again, and during the dorment time they might have been dispersed to other location. Sexual reproduction provides a generation of novel genotypes through recombination. Individuals that hatch from epphippia not only have the potential to survive over unfavorable conditions, but also the sexual process increases the probability that some of the new genotypes produced are better adapted to novel environmental conditions in which the eggs will hatch.

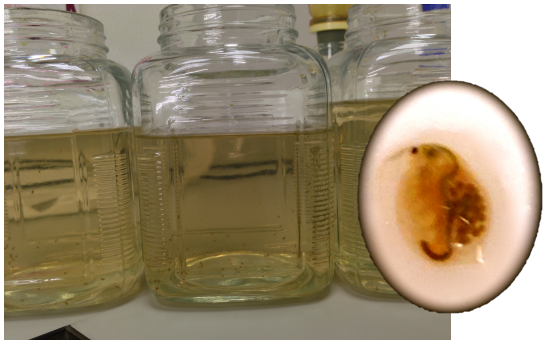


Figure 1.5. *Daphnia magna* in laboratory culture jars (left) and adult female Daphnia under light microscope (right). *Source:* Fabianne Ribeiro

Newborns go through five events of moulting to become mature, after which they continue to grow. Moulting also happens after each occasion of brood release. Daphnids feed on live and dead suspended matter, including protozoa and bacteria, but mainly on phytoplankton.

The small size and short life cycle of *Daphnia*, in addition to its ecological relevance and distribution are the main features attributed to the large use of the species as model organism in ecotoxicological assessments.

Danio rerio (Francis Hamilton, 1822)

Danio rerio, commonly known as zebrafish belongs to the cyprinidae family and it is originated from the Hymalaian regions, with some records also in the south of Asia (Spence et al., 2006). This species has been successfully used in ecotoxicological studies as a model species for vertebrate testing: their relatively short life-cycle, adult size, and embryo development are among the features that make this species reliable as an alternative for animal testing (Lammer et al., 2009). *Danio rerio* can be easily maintained under laboratory cultures for years,

which facilitates their matching and reproduction, with a great number of eggs, suitable for testing. The zebrafish eggs develop outside of the parental body, and the egg transparency allow the observation in time of their embryonic development and the detection of developmental abnormalities caused by the testing substance.

Figure 1.6. *Danio rerio* under laboratory cultures in the Zebrafish facility situated at the Department of Biology from the University of Aveiro (left) and a *Danio rerio* embryo at under stereomicroscope, at 48h post fertilization (right corner). *Source*: Rhaul Oliveira & Fabianne Ribeiro

Carassius auratus auratus (Linnaeus, 1758)

The common goldfish *Carassius auratus, o*riginally native from Eastern Asia is a successful invasive species in Europe, North and South America, New Zealand and Australia (Lorenzoni, Dolciami, Ghetti, Pedicillo, & Carosi, 2010). *Carassius auratus* belongs to the carp family (Cyprinidae) and as most of the species of this family the feeding habits are based on invertebrates and plants which are believed to be associated with the lack of teeth and stomach. Reproduction often occurs after a significant change in temperature, usually during spring. *Carassius*

auratus is an oviparous fish species; the females carrying eggs are chased by males, which prompt them to release the eggs. After released, the eggs are prone to attach to aquatic vegetation and hatch occurs within 72 hours. The goldfish fry starts to assume definitive morphological characteristics within one-week period, as a response to the predation pressure. *Carassius auratus* was chosen in this thesis as a model organism because of their small size, for having ecological relevance as a successful invasive species in Europe, and also because this species is being increasingly used in ecotoxicity testing. In addition to that, *C. auratus* potentially inhabits similar environments such as *Raphidocelis subcapitata* and *Daphnia magna*, a fact that gives the trophic chain study developed in chapter 5 of this thesis, a certain environmental relevance.

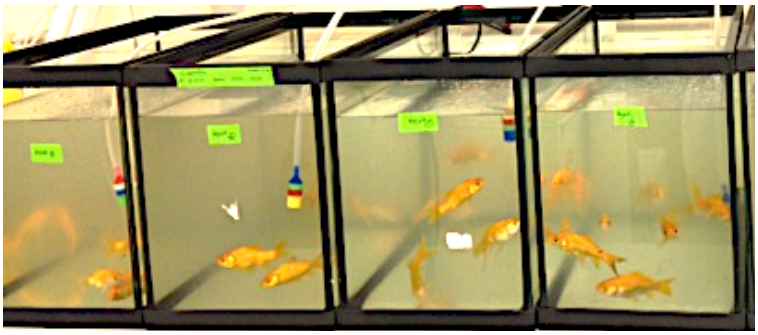


Figure 1.7. *Carassius auratus* juveniles during acclimation period under laboratory conditions. *Source*: Fabianne Ribeiro

1.7 Objectives and framework of the thesis

Due to the recent expansion of nanotechnology and increase presence of nanoparticle containing products in the market, there is a need for the risk assessment that nanoparticle will represent to the environment. Researchers from diverse areas such as chemistry, physics, biology, material engineering, environmental sciences, etc. have joint efforts in order to develop a robust database concerning nanoparticles production, characteristics, fate and effects in the environment. This study was partly supported by NanoFATE (Nanoparticle Fate Assessment and Toxicity in the Environment), a FP7-EU-funded integrated research project that aims at gathering information regarding ecotoxicity testing procedures and effects of some of the most used nanoparticles in both aquatic and terrestrial species, and also partly supported by the Portuguese National funding through FCT-Fundação para a Ciência e Tecnologia, within the research project FUTRICA— Chemical Flow in an Aquatic TRophic Chain (FCOMP-01-0124-FEDER-008600; Ref. FCT PTDC/ AAC-AMB/104666/2008)

In this thesis I attempt on answering some questions concerning silver nanoparticles (AgNP) effects and bioaccumulation on three aquatic species that have critical importance in an aquatic food chain. The final aim was to determine whether AgNP are transferred from a basal trophic level (in this study, represented by the green algae *Raphidocelis subcapitata*), to a primary consumer (*Daphnia magna*) and finally to a secondary consumer fish here represented by the species *Carassius auratus*.

In order to achieve the final objective of this study, the research was divided into 4 different tasks, which are reported in this thesis in the chapters 2 to 5.

Chapter 2 addresses the toxic effects of silver nanoparticles in comparison with silver nitrate (used as an ionic control) on the three species of my model trophic chain (*Danio rerio* is used over *Carassius auratus* in this chapter; see chapter 6 for the justification on that). The effects of silver nanoparticles and ions are reported on the basis of nominal Effective-Concentration, i.e., EC_{50} for each of the test species and their relevant endpoints. This study has already been published in the journal Science of the Total Environment (2014) 466–467: 232–241.

Chapter 3 aimed at relating the bioconcentration of AgNP by *R. subcapitata* to the size of the different Ag fractions in solution. The experimental design included the separation of the algae media containing AgNP and $AgNO_3$ into three different size fraction, namely Ag_{tot}, Ag_{dis} and Ag_{ionic}. The results are reported in terms of Bioconcentration Factor (BCF) for each of the fractions, and were compared between them with the objective of establishing a relationship between bioavailability of Ag (represented by the size of the fractions) and bioconcentration by algae.

Chapter 4 deals with bioaccumulation of silver nanoparticles in *Daphnia magna*, under different exposure routes, i.e., waterborne, diet and both water and dietary routes. Information on toxicity from chapter 2 was used in this chapter to expose *R. subcapitata* to AgNP before supplying it as food to *D. magna*. All routes of exposure to AgNP were also reproduced for $AgNO_3$ in order to detect differences between nanoparticle and ionic exposure in terms of Bioaccumulation Factors (BAF´s). The objective of this chapter was to identify which route of exposure represents a potential worse-case scenario for the bioaccumulation of AgNP in *Daphnia magna*.

Chapter 5 regards the trophic transfer of silver nanoparticles from a baseline producer species *R. subcapitata* to the secondary consumer *C. auratus*. The experimental setup of this investigation was based on previous results that were obtained in the earlier studies. Therefore, I tried to simulate an aquatic trophic chain, in which Daphnia was exposed to waterborne AgNP and fed with Ag-contaminated algae, and fish were exposed to waterborne AgNP and fed with Ag-previously contaminated Daphnia, as represented in figure 1.8. The time of exposure of both algae and Daphnia to AgNP and $AgNO_3$ was chosen based on the findings from chapter 2 and 3. Finally, in this chapter, the toxicokinetics of Ag was reported in the basis of each organ separately as well as biomagnification factors (BMF) were calculated for each of the fish organs in order to determine whether biomagnification had occurred.

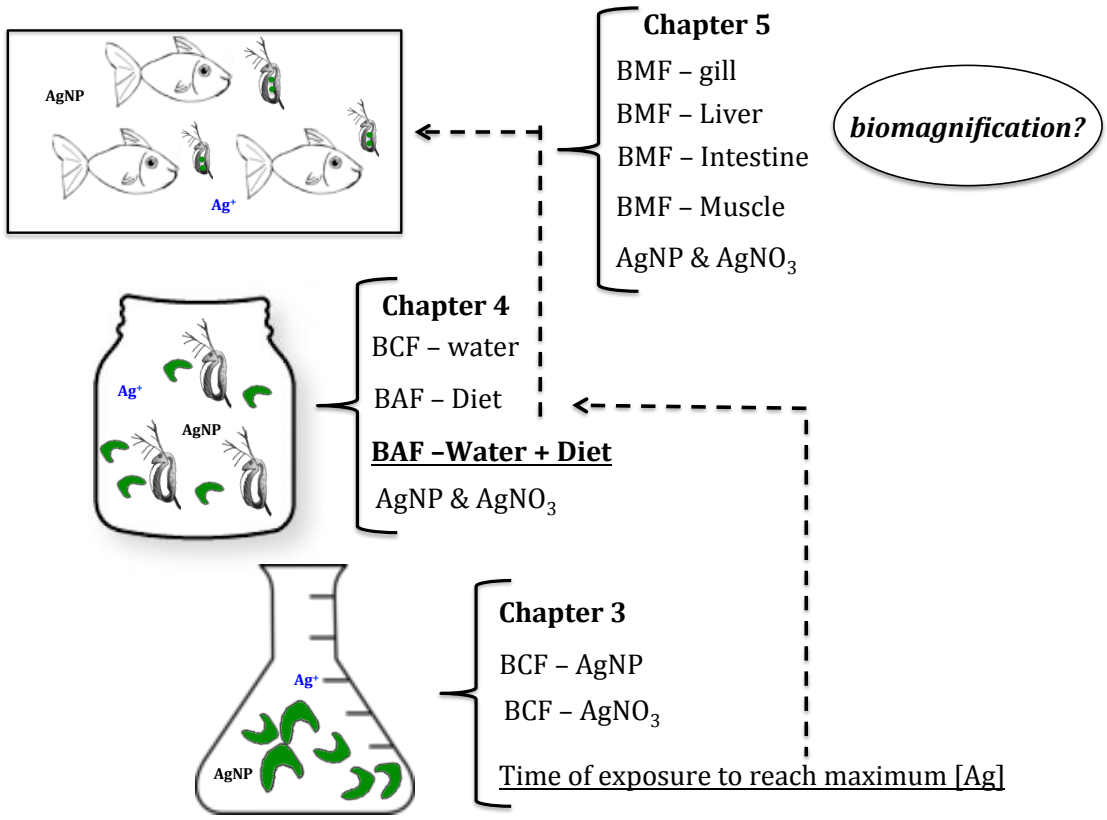


Figure 1.8. Conceptual framework of the bioaccumulation studies with *Raphidocelis subcapitata*, *Daphnia magna* and *Carassius auratus.*

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Chapter 2

Toxicity of silver nanoparticles to *Raphidocelis subcapitata*, *Daphnia magna* and *Danio rerio*

Source: Fabianne Ribeiro

2 Silver nanoparticles and silver nitrate induce high toxicity to *Raphidocelis subcapitata*, *Daphnia magna* and *Danio rerio*

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Abstract

Silver nanoparticles (AgNP) have gained attention over the years due to the antimicrobial function of silver, which has been exploited industrially to produce consumer goods that vary in type and application. Undoubtedly the increase of production and consumption of these silver-containing products will lead to the entry of silver compounds into the environment. In this study we have used *Pseudokirchneriella subcapitata*, *Daphnia magna* and *Danio rerio* as model organisms to investigate the toxicity of AgNP and $AgNO_3$ by assessing different biological endpoints and exposure periods. Organisms were exposed following specific and standardized protocols for each species/ endpoints, with modifications when necessary. AgNP were characterized in each test-media by Transmission Electron Microscopy (TEM) and experiments were performed by Dynamic Light Scattering (DLS) to investigate the aggregation and agglomeration behavior of AgNP under different media chemical composition and test-period. TEM images of AgNP in the different test-media showed dissimilar patterns of agglomeration, with some agglomerates inside an organic layer, some loosely associated particles and also the presence of some individual particles. The toxicity of both $AgNO_3$ and AgNP differ significantly based on the test species: we found no differences in toxicity for algae, a small difference for zebrafish and a major difference in toxicity for *Daphnia magna*.

2.1 Introduction

The antimicrobial activity of silver nanoparticles make them useful in a wide range of products varying from medical devices and antimicrobial control systems to consumer goods such as clothes and personal hygiene products. The increase on production and consumption of silver-containing products will certainly lead to the release of AgNP into the environment. This can happen at any stage of the product life-cycle: production, transport, storage, usage and disposal. The Woodrow Wilson Database (http://www.nanotechproject.org) listed 259 products containing AgNP present in the market in 2010 (Fabrega et al., 2009). The list for 2012 has already 54 new Ag containing products, indicating a continuous growth of production. Some of the main routes of entry of AgNP into the aquatic environment are from the washing of Ag-containing clothes (Benn and Westerhoff, 2008), or from the use of cosmetics and hygiene products, such as tooth paste and soap as well as disposal of food containers. The prediction for 2010 was that 15% of Ag found in European waters would be released from biocidal plastics and textiles (Blaser et al., 2008). In natural freshwaters Ag can be found as silver chloride (AgCl), silver sulfide (Ag2S) or bounded to organic matter. The ionic form Ag^{+} is recognized as being the most toxic to aquatic organisms (Hogstrand and Wood, 1998) even though there might be some other Ag species that are also available to the organisms (Campbell et al., 2002). Silver nanoparticles differs from the macro-scale Ag counterpart in their distinctive properties such as enhanced surface Raman scattering (Elechiguerra et al., 2005), high thermal and electrical conductivity, catalytic activity and non-linear optical behavior; some of these size-specific properties

combined with the transport enhancement provided by the small size could induce some boosted toxicity when compared to other Ag species. Furthermore, there are a number of processes that are specific for AgNP compared to the dissolved counterpart. The dissolution process of AgNP, consisting on the release of dissolved Ag^+ ion from the particle, is crucial for an adequate assessment of the exposure and hazard of AgNP (Nowack et al., 2012). Dissolution will be dependent on the chemical composition of the media, temperature, concentration of nanoparticles in solution, pH and coating of nanoparticles (Liu and Hurt, 2010). Another process that occurs once AgNP reaches the aquatic environment is aggregation and heteroaggregation, i.e. the collision and attachment with another AgNP or with another type of particle, respectively. These two processes are dependent on the media composition, the particles coating and the number (concentration) of particles. Due to the large production and diverse applications of AgNP it is expected that particles differing in size, shape and coating material will be found in the environment (Evanoff and Chumanov, 2005). The Predicted environmental concentration (PEC) for Ag in the aquatic environment ranges from 0.03 μg/L to 1 μg/L (Tiede et al., 2009; Mueller and Nowack, 2008). Ag is one of the most toxic metal found in natural waters systems and considering that situation and the lack of information on presence and behavior of AgNP in the environment, there is a need to assess the toxicity of AgNP with different characteristics, to aquatic species and which will have a contribution on building the knowledge concerning their safe production and application. The dissolution of metal NP appeared to be the main factor leading toxicity of NP to test organisms in many cases as a few studies have shown so far. Zhao and Wang (2011) reported that AgNP per se had no effect on

Daphnia magna survival, while the LC_{50} found for silver nitrate ($AgNO_3$) was as low as 2.51 μg/L. The authors related the toxicity to the released Ag ions. In another study by Navarro et al. (2008b) the toxicity of AgNP to the green algae *Chlamydomonas reinhardtii* was evaluated and the inhibition on photosynthesis was considered to be mediated by the release of Ag ions. On the other hand, Griffitt et al. (2008) found that toxicity of nanosilver to *Danio rerio* and *Daphnia pulex* was not attributed to particle dissolution, suggesting that particular nanometals have an intrinsic property that exerts toxicity. Furthermore, Asharani et al. (2008) observed a higher toxicity of AgNP on the development of zebrafish (*D. rerio*) embryos when comparing to the dissolved Ag form, possibly due to the penetration of nanoparticles through the chorionic membrane and their interactions with the embryos tissues in formation. There is an indicative divergence on the toxic effects of nanoparticles concerning the chemical characteristics of the particle, the study species and endpoints. Therefore, the objective of the present study was to address the biological effects of small-sized AgNP (original size 3–8 nm) in comparison with $AgNO_3$ exposures on three aquatic species: the unicellular microalgae *Raphidocelis subcapitata*, the cladocera *Daphnia magna* and the fish *Danio rerio*. Besides being standardized, which allows us to have a more reliable comparison of results with already published data, these species are also representative of three different levels of an aquatic trophic chain, which might enable the linking of single species biological effects with possible ecological implications. In addition, to our knowledge, this AgNP (ranging from 3 to 8 nm) is among the smallest AgNPs used in ecotoxicological testing and, as size has been considered one of the characteristics that rules toxicity, it will be crucial to assess and compare its

toxicity with the toxicity induced by other AgNPs.

2.2 Material and methods

Test-organisms

The green algae *R. subcapitata* was maintained in laboratory cultures in the Woods Hole MBL culture medium. *R. subcapitata* was cultured under constant light conditions and temperature of 20 °C ± 1 °C. Exponential growing cells were used for the bioassays and 7-days old cultures were renewed by inoculation in a new MBL medium. *Daphnia magna* clone K6 was maintained in ASTM (American Society for Testing and Materials) medium at temperature of 20 °C ± 1 °C, under a 16 h:8 h light–dark cycle inside a controlled-temperature chamber. The culture medium was renewed and daphnids were fed three times per week with the micro-algae *R. subcapitata*. Neonates used for toxicity tests were from the third to the fifth brood, and the sixth brood was used to start a new culture. The zebrafish (*D. rerio*) culture facility, located at University of Aveiro, Department of Biology (Portugal) provided the eggs for the Fish Embryo Toxicity (FET) test. Organisms are maintained in carbon-filtered water added with salt "Instant Ocean Synthetic Sea Salt" and the culture conditions are: 25.0 ± 1 °C, 16 h:8 h light– dark photoperiod cycle, conductivity: 550 ± 50 µS, pH: 7.5 ± 0.5 and dissolved oxygen 95% saturation. Adult fish are fed twice daily with commercially available artificial diet (ZM 400 Granular) and brine shrimp.

Chemicals

Silver nitrate was purchased from Sigma-Aldrich as a crystalline powder, 99% purity CAS 7761-88-8. AgNP with an alkane coating and with a small (3–8 nm) size range were supplied by AMEPOX (Poland). They were dispersed in water with initial concentration of 500 mg/L. All test solutions were prepared by dilution of the initial dispersion of AgNP in the culture media to the desired concentration.

Analytical measurements

Media samples from all tests were digested for total Ag measurements. Twenty-four hours prior digestion, concentrated H_2O_2 and HCl were added to 5 mL of each sample (bringing its concentration to 5% and 1% v/v in the sample, respectively). After this, samples were transferred to Teflon beakers and allowed to evaporate (boiling was avoided) on a hot plate until 0.5–1 mL volume was remained. After evaporation, aqua-regia ($1HNO_3$: 3HCl) was added to the samples and heated without boiling for 1 h. All samples were cooled to room temperature and then transferred to 50 mL falcon tubes and diluted to 45 mL with 1% HCl solution. Total Ag was measured by Graphite-Furnace Atomic Absorption Spectroscopy (GF-AAS).

Characterization of nanoparticles

Agglomeration experiments and TEM images of silver nanoparticles in test media

The Z-average hydrodynamic diameter of the AgNP at 10 mg/L was measured over 2 weeks (the maximum length of many toxicity tests) using a Malvern Zetasizer. Up to 18 replicates were taken to gain a robust value for the mean-z-average hydrodynamic diameter. Aggregation/agglomeration experiments on test-media were also performed by a Malvern Zetasizer equipment and Zetasizer software 6.20. Short and long term experiments were conducted. For the short (minutes) experiments, the initial stock nanoparticles suspension was diluted with the media and with milli-Q water to the desired concentrations in normal DLS cuvettes and inserted immediately in the instrument. The measurement was started at a fixed attenuator and measurement position to avoid the optimization time, the correlation time was set to 2 s and 120 data points were generally obtained. For the long term experiments (days) the first measurement (day zero) was obtained by creating an average result from the short term data points. The cuvettes were stored in the dark and three measurements were performed (3 runs of 20 s each) in the following days. To evaluate the effect of particles sedimentation, the samples were shaken after performing the measurement and a new measurement was done. Derived count rates are included in the long term experiments to compare the capacity of the remaining particles (large and small) to scatter light. For TEM imaging, the initial suspension of AgNP (500 mg/L) was diluted to 0.1 mg/mL in each of the test media, and a drop of this suspension was deposited on a holey carbon coated Cu TEM grid and dried at room temperature

for several hours before examination. Experiments were carried out on a JEOL 2010 analytical TEM which has a resolution of 0.19 nm, an electron probe size down to 0.5 nm and a maximum specimen tilt of ±10° along both axes. The instrument is equipped with an Oxford Instruments LZ5 windowless energy dispersive X-ray spectrometer (EDS) controlled by INCA.

Toxicity tests

Algae growth inhibition test

The growth rate inhibition of *R. subcapitata* was assessed following a method that combines both the OECD 201 guideline (OECD, 2006) with adaptations and the methodology described in Eisentraeger et al. (2003) for the 24-well microplates experiment. Exponential growing cells were taken from the algae culture counted on the microscope using a newbauer camera. A starting concentration of $5x10^4$ algae cells/mL was incubated in each well containing serial dilutions of the starting solution of $AgNO_3$ and AgNP on MBL medium. Each microplate had samples from the control treatment, blank and all concentrations, following the disposition described in Eisentraeger et al. (2003). Three replicates were performed per concentration for both $AgNO_3$ and AgNP. Plates were incubated in a light-temperature controlled chamber at 25 °C for 72 h with a photoperiod of 16 h:8 h light–dark. Every 24 h the plates were manually shaken to re-suspend any settled cells and after 72 h, a sample from each well was read in a spectrophotometer at 440 nm. The concentration of cells in each well was calculated by the equation:

$$C = -17{,}107.5 \times ABS \times 7{,}925350 \quad (1)$$

Where:

C is the algae concentration in cells per milliliter and ABS is the absorbance measurement, at 440 nm. The average of the specific growth rate for each period was obtained as the biomass increase after the 72 h, by the following equation:

$$\mu_{i-j} = \frac{\ln B_j - \ln B_i}{t_{j-t_i}} \quad (2)$$

where μ_{i-j} is the average specific growth rate from time i to time j, ti is the initial time of the exposure period, t_j is the final time of exposure, C_i is the biomass (concentration of cells) at time i and C_j is the biomass at time j. Percentage inhibition of growth was calculated as:

$$\%I_r = \frac{\mu_T - \mu_c}{\mu_c} \times 100 \quad (3)$$

where % I_r is the percent inhibition in average specific growth rate; μ_C is the mean value for average specific growth rate (μ) in the control group and μ_T is the average specific growth rate for the treatment replicate.

Acute and chronic tests with D. magna

Immobilization tests were performed according to the OECD 202 guideline (OECD, 2004). *Daphnia magna* neonates (<24 h) were exposed in 50 mL glass vials containing ASTM with different concentrations of $AgNO_3$ and AgNP. The test duration was of 48 h without food and under culture conditions, i.e., with photoperiod and controlled temperature. In addition, an immobilization test in which food was provided was performed for both $AgNO_3$ and AgNP to detect

differences in the LC_{50} between the conditions of presence and absence of food. After 24 h and 48 h, the immobilization of daphnids was recorded. They were considered immobile if they were not capable to swim after gentle agitation of the test-vial or even if they could still move their antennae.

Nominal concentrations employed for immobilization assays without food were from 0.2 µg/L to 20 µg/L of Ag^+ and AgNP and in the presence of food, measured concentrations varied from 1 µg/L to 5 µg/L for Ag^+ and from 15 µg/L to 105 µg/L for AgNP. Daphnia in the control were exposed to ASTM only, and the ones in the positive control were exposed to the highest concentration of NP protection layer present in the test. Five replicates were performed per treatment and five daphnids were exposed in each vial, making a total of 25 organisms per treatment. At the end of the test, pH and oxygen concentration in the test-medium were measured. Reproduction was assessed according to the OECD 211 guideline (OECD, 1998) with adaptations regarding to the chemical stability of dispersed AgNP in the test media. Less than 24 h-old neonates were exposed to a range of concentrations of $AgNO_3$ and AgNP. Organisms were maintained individually in glass vials containing 50 mL of ASTM moderated hard media with seaweed extract and food (supplied as *R. subcapitata*). The reproduction test lasted for 21 days and the test media was renewed daily. Nominal concentrations of $AgNO_3$ (calculated as Ag^+) and AgNP were: 0.1, 0.5, 1.0, 2.0 and 5.0 µg/L. As described above, a negative and a positive control were also performed. Experimental design included ten replicates per treatment.

The feeding inhibition bioassay was conducted following the method described in Allen et al. (1995). Less than 24 h-old neonates were separated from the main cultures and kept at same conditions as cultures until the release of fourth moult.

At this stage, organisms were exposed to ASTM at different concentrations of $AgNO_3$ and AgNP and a fixed concentration of 5 x 10^5 cells/mL of the microalgae *R. subcapitata*, provided as food. This test was conducted in glass beakers containing 100 mL of test solution. The experimental design had 5 replicates per treatment including the control, and five daphnids per replicate. Moreover, an additional replicate with contaminated media and algae was conducted for each treatment to be used as the initial concentration of algae on the feeding calculations. Another control containing ASTM media and AgNP at the highest concentration tested was checked for the influence of AgNP spectral absorption on the measurements. Concentrations employed were 0.1, 0.5, 1.0 and 2 µg/L for $AgNO_3$ and 5, 10, 20 and 25 µg/L for AgNP. Feeding inhibition of *D. magna* was assessed for 24 h in the dark where daphnids were allowed to feed in a contaminated media, after which they were transferred to a "clean media" (not contaminated) with algae and allowed to feed for 4 h in the dark. This additional 4 h was conducted to assess the feeding activity on the post-exposure period. After the 24 h-exposure and the 4 h-post exposure times, algae concentration was measured by spectrometry at 440 nm and the concentration of algae cells in the media was calculated using Eq. (1). The feeding rate of *D. magna*, for each replicate, was calculated by the equation:

$$\mu_{feeding} = (\mathrm{CBR} - \mathrm{Cx}) \div (\mathrm{t} \times \mathrm{n})$$

where: CBR is the initial concentration of algae cells in each vial, Cx is the final concentration of cells in the vial of the same treatment, t is the time of exposure and n is the number of replicates at each concentration.

FET (fish embryo toxicity)

This test was based on the OECD draft guideline on Fish Embryo Toxicity (FET) Test (OECD, 2012). Zebrafish eggs were collected 30 min after natural mating, rinsed with water and selected under a stereomicroscope for its viability for the assay i.e., fertilized and not showing abnormal cleavage. Eggs with an opaque appearance were considered dead and not included in the experiment. Zebrafish eggs were distributed in a 24 well plate with containing 3 mL of the culture water, with several dilutions of $AgNO_3$ and AgNP: for AgNP, 10; 25; 50; 100 and 250 µg/L; for $AgNO_3$, 0.5; 1.0; 2.5; 5 µg/L. 30 replicates of each concentration were performed, i.e. one egg per well as one replicate. Negative and positive controls were also conducted. The eggs were incubated at 25 °C for 96 h. Every 24 h they were checked under the stereomicroscope and the main endpoints recorded were survival, hatching, tail detachment, and presence of edema.

Data treatment

Data from growth inhibition of *R. subcapitata*, feeding inhibition and reproduction of *D. magna* were analyzed by a one-way ANOVA (Systat, 2006). Data sets that followed a normal distribution were analyzed by the Dunnet's method to check for differences between treatments and the control ($p = 0.05$). For data that failed the normality test and data transformation procedures, a non-parametric Kruskal–Wallis test was used and the multiple comparisons with Dunn's method conducted ($p = 0.05$). The EC_{50} values for all endpoints were calculated by non-linear regression, a logistic 3-parameter equation (Systat, 2006). Lethal concentrations (LC_{50}) were obtained by probit analysis, with the MINITAB software (Minitab, 2003).

2.3 Results

Characterization of AgNPs

Aggregation experiments and TEM images

The hydrodynamic diameter of the AgNPs was reasonably stable for the duration of the experiment at 127–132 nm with an error less than ±4 nm (Fig. 2.1). These agglomerates and diameters showed no definite changes during the experiment and therefore they are likely to be stable for the duration of 2 weeks. Moreover, Z-average size of AgNP at 1 mg/L (stock solution) was followed over three days in all test media that were used in this study; this concentration was used because it is the lowest concentration that gave statistically significant results from the DLS analysis. Fig. 2.3 shows that in ASTM, the zeta-average diameters of particles agglomerates at day zero (labeled in the figure 2.3 as day 1) was approximately 80 nm and after one day of experiment the zeta average has increased to ~200 nm, denoting that large agglomerates are starting to impact the scattered intensity weighted diameter (zeta-average). Agglomerates appeared to be larger after 3 days, reaching 350 nm. In MBL at day zero, average size of agglomerates was approximately 100 nm and after three days the agglomerate size varied between 200 and 250 nm. In zebrafish water, agglomerates were approximately 100 nm at time zero, and reached 200 nm after 3 days of experiment.

The AgNP used in this study are an example of a particle used in the production of special materials such as conductive adhesives for electronics and microelectronics industries (AMEPOX, Poland) and have a nominal primary

particle size of 3–8 nm and a paraffin coating of approximately 18 wt.%. TEM measurements of primary particle size of individual particles gave a diameter of 7.5 ± 1.7 nm measured on >50 particles. In addition to well dispersed primary particles there is a large number of paraffin covered aggregates of approx. 30–100 nm diameter (Fig. 2.2A).

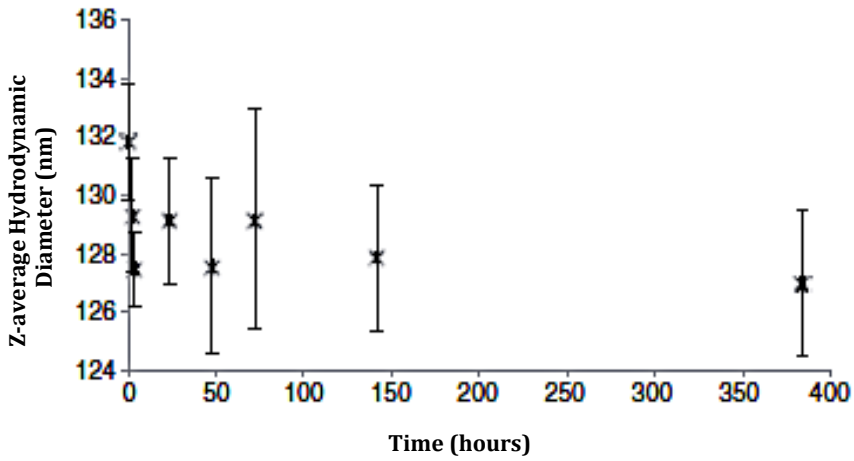

Figure 2.1. Z-average hydrodynamic diameter (nm) in ultra-pure water at 10mg/L.

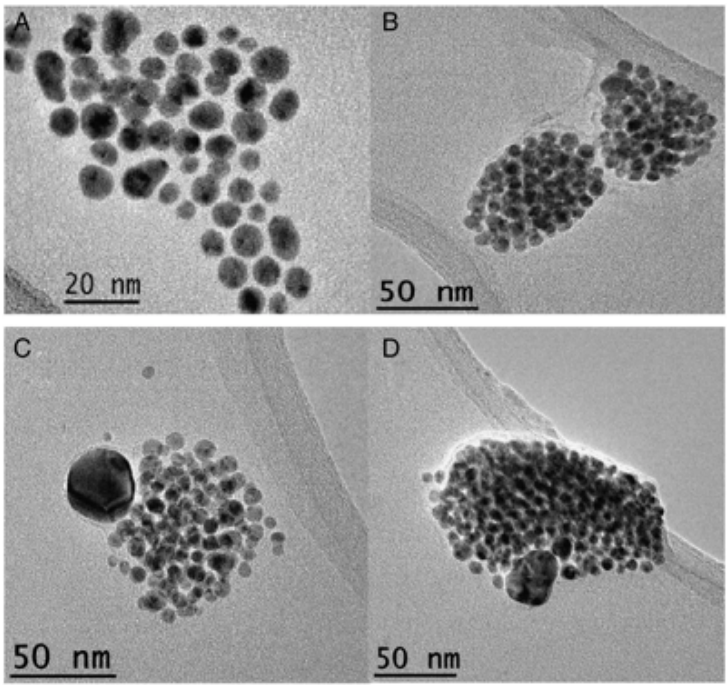

Figure 2.2. TEM images of silver nanoparticles. A: Initial suspension of AgNP in water, with particles ranging in size from 3 to 8 nm. B: AgNP dispersed in ASTM media, showing nanoparticles agglomerates and the organic film on the outside. C: AgNP in MBL media, with great variability of primary particle size, ranging from 2 to 50 nm. D: Ag–NP dispersed in zebrafish water, showing that particles were wrapped in an organic layer.

When in media like ASTM, Woodshole MBL or zebra fish water the coating of the agglomerates has a tendency to interact with chemicals in the media. The agglomerates break up, releasing more primary particles into the media. These individual particles can then dissolve and/or react with components of the media. This effect is least pronounced in the ASTM medium, where few changes with respect to primary particle size can be detected (Fig. 2.2B). In MBL and zebrafish water however, the range of individual particle size is extended to 2–50 nm. There was a wider variation of particle size with primary particles from approximately 2–3 nm to approximately 20–50 nm suggesting the possibility that a dynamic process was undergoing involving dissolution of the initial

particles and formation of larger ones. In some cases, several primary particles appear to have welded together and form irregular shaped particles. The EDX from the metallic particles showed that there is extremely little sulphur related to the Ag particles and it can be assumed that the particles are metallic Ag at this stage. In the zebrafish water, particles appeared mostly in agglomerates of a few particles up to approximately 100 nm, a number of individual particles and/or very loosely associated particles could be seen as well (Fig. 2.2D).

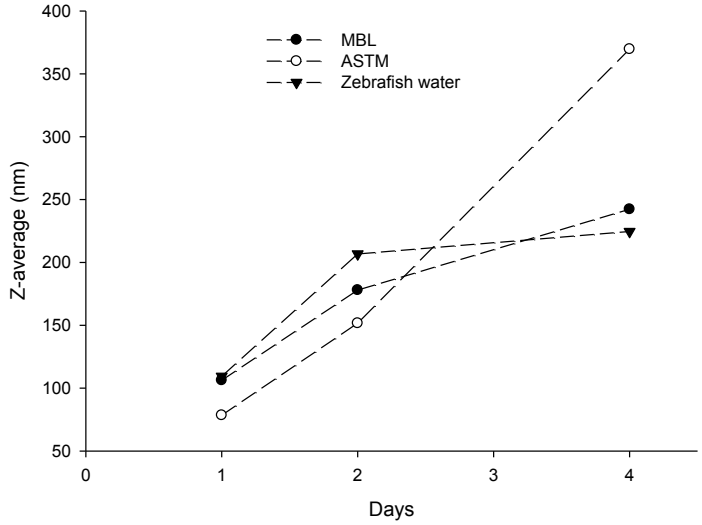

Figure 2.3. Zeta average diameter of silver nanoparticles at 1 mg/L followed by 4 days period in each test-media. The test-medias are indicated by the different dots in the figure.

Chemical analysis

The average percentage recovery of Ag in the all the toxicological media samples was 91% (SE = 2.61). The results and discussion are based in nominal concentrations.

Toxicity tests

The EC_{50} value of $AgNO_3$ for the growth inhibition of *R. subcapitata* (after 72 h) was 33.79 µg/L (SE = 2.96). Significant differences from the control growth rates were detected at the concentration of 50 µg/L of Ag^+, where the growth inhibition is almost 100%. At 100 µg/L of Ag^+ the growth inhibition was very similar from the 50 µg/L exposure, whereas at 125 µg/L of Ag^+, growth inhibition decreased. For AgNP effects on growth inhibition of *R. subcapitata*, the EC_{50} was 32.40 µg/L (SE = 2.09), and differences in the growth rate relatively to the control were detected also from 50 µg/L of AgNP and above ($p < 0.05$, Dunn's method), being the highest concentrations also lethal to the algae, with growth inhibition percentages higher than 100%. By comparing the percentage inhibition on specific growth rate induced by AgNP and $AgNO_3$ we observed that at 25 µg/L, AgNP is twice toxic than $AgNO_3$ and at 50 µg/L, AgNP induces a lower percentage of growth inhibition when compared to $AgNO_3$ (Fig. 2.4).

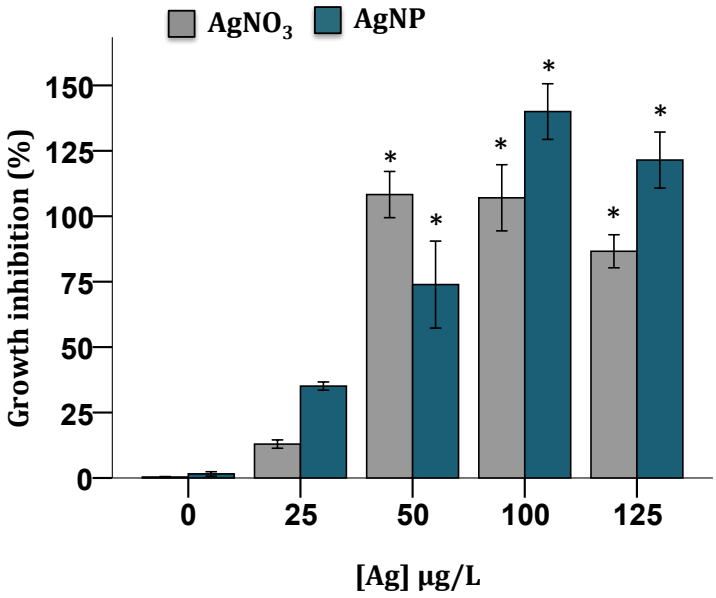

Figure 2.4. Growth inhibition of the microalgae *Raphidocelis subcapitata* after a 72h exposure to silver nitrate and silver nanoparticles. Asterisks indicate significant differences in growth rate compared to control group. Dunn's test, $p < 0.05$.

The immobilisation of *D. magna* was recorded after 24 h and 48 h of exposure to $AgNO_3$ and AgNP. In the absence of food, the 24 h-LC_{50} of AgNP and $AgNO_3$ were 11.41 µg/L (SE = 0.98) and 1.36 µg/L (SE = 0.09), respectively, while the 48 h-LC_{50} for AgNP and $AgNO_3$ was 11.02 µg/L (SE = 0.88) and 1.05 µg/L (SE = 1.85), respectively. When acute tests were conducted with the addition of food, the 24h-LC_{50} for AgNP and $AgNO_3$ increased to 81.84 µg/L (SE = 2.17) and 3.71 µg/L (SE = 0.08), respectively. In the presence of food, the 48 h exposure resulted in a LC_{50} value for $AgNO_3$ of 3.38 µg/L (SE = 0.08) and 72.0 µg/L (SE = 1.85) for the AgNP exposure (Table 2.1). The feeding activity of *D. magna*, measured as feeding rates after 24 h of exposure to increasing concentrations of $AgNO_3$ and AgNP, was affected in a concentration dependent way for both exposures.

For $AgNO_3$, the 24 h-EC_{50} for feeding inhibition was 2.03 µg/L (SE = 0.35), and

the feeding rate of *D. magna* was only lower than the control at 2 µg/L of $AgNO_3$ (Dunn's method) (Fig. 2.5a). For AgNP, the EC_{50} for feeding inhibition was 13.64 µg/L (SE=1.85). For AgNP exposure, the feeding rates were significantly affected at 10 µg/L and 20 µg/L (Dunnet's method) (Fig. 2.5b).

Table 2.1. Comparative values of 24 h and 48 h LC_{50} of AgNP and $AgNO_3$ in the presence and absence of food in the media.

	AgNP		**$AgNO_3$**	
	With food	*Without food*	*With food*	*Without food*
24h-LC_{50}	81.84 (2.27)	11.41 (0.98)	3.71 (0.08)	1.36 (0.09)
48h-LC_{50}	72.00 (1.85)	11.02 (1.85)	3.38 (0.08)	1.04 (1.84)

Table 1.2. Comparative values of EC_{50} for nominal concentrations of silver nitrate and silver nanoparticles for all species and endpoints

	Test-species	**Endpoint**	**EC_{50} (µg/L)**
AgNP	*Pseudokirchneriella subcapitata*	Growth Inhibition	32.4 (2.09)
	Daphnia magna	Immobilisation	10.2 (41.0)
	Daphnia magna	Feeding inhibition	13.64 (1.8)
	Daphnia magna	Reproduction	1.0 ($2.4X10^{-22}$)
	Danio rerio	Mortality	128.4 (7.1)
$AgNO_3$	*Pseudokirchneriella subcapitata*	Growth Inhibition	33.79 (2.9)
	Daphnia magna	Immobilisation	1.05 (1.85)
	Daphnia magna	Feeding inhibition	2.03 (0.35)
	Daphnia magna	Reproduction	0.38 (1.10)
	Danio rerio	Mortality	78.32 (5.79)

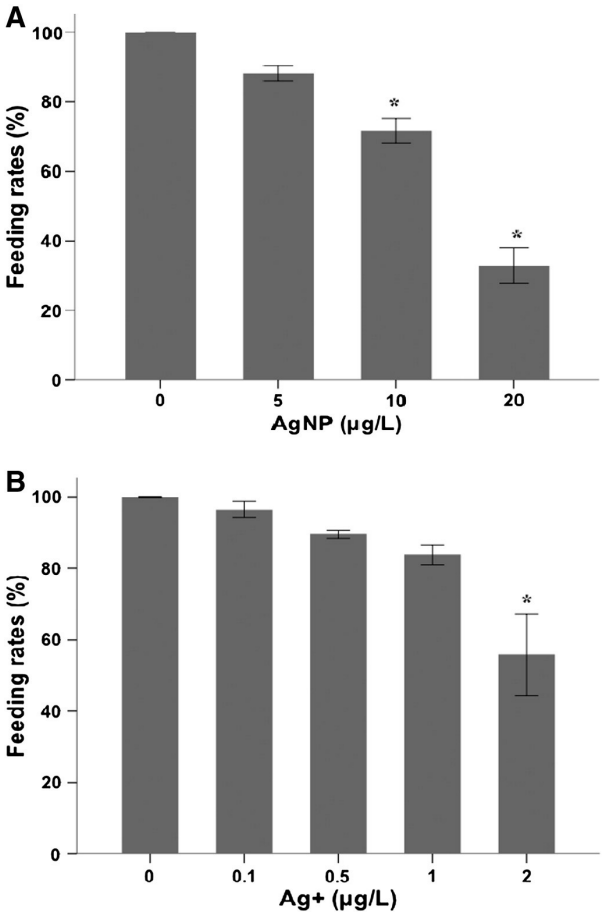

Figure 2.5. Feeding rates of *Daphnia magna* (cells/mL/Daphnia) expressed as percentage of the control upon exposure to silver nanoparticles (upper) and silver nitrate (bottom). Concentrations are nominal values expressed as µg Ag/L. Asterisks represents statistical differences from the control obtained by Dunnet's test.

For the reproduction response of *D. magna*, the positive control of AgNP (capping agent) was different from the negative control (ASTM) considering the total number of neonates produced by the end of the 21d of exposure (t-test, p = 0.004) with a percentage of difference of 96.1% in relation to the negative control. Therefore, treatments were compared to the positive control. For $AgNO_3$, the 21-day EC_{50} was 0.385 µg/L (SE = 0.10) and for AgNP exposure, the 21 day EC_{50} for reproduction was 1.0 µg/L (SE = 2.48). A decrease on the mean number

of neonates produced per female was already observed from 0.5 µg/L of AgNO3 onwards (P ≤ 0.001 Dunn's method) and 1 µg/L for AgNP (P ≤ 0.001 Dunnet's test) (Fig. 2.6). Growth of daphnids exposed to $AgNO_3$ and AgNP was measured by the difference between the size of newborns and adults (after 21d exposure), and it was not significantly affected neither by $AgNO_3$ nor AgNP ($p > 0.05$) (data not shown).

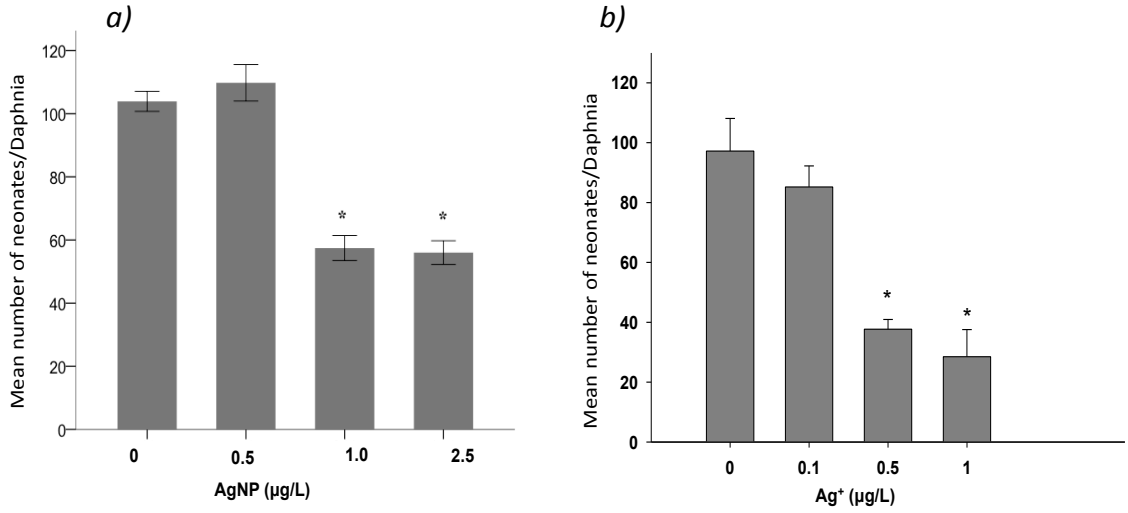

Figure 2.6. Reproduction response of *Daphnia magna* after 21 days of exposure to *a*) silver nanoparticles and *b*) silver nitrate. Concentrations of silver are in nominal values. Asterisks stands for statistical differences from the control (Dunnet's method).

FET (fish embryo toxicity)

The endpoints recorded on the zebrafish toxicity test were similar for both $AgNO_3$ and AgNP. Control eggs (both culture water and capping agent control) exhibited normal development and no mortality was observed. By the end of 48h post fertilization, all control eggs were hatched and the larvae had a normal development (Fig. 2.9 top line). $AgNO_3$ proved to exert higher toxicity on the

hatching rate of the eggs than AgNP (Fig. 2.8). The nominal 96 h-LC_{50} for $AgNO_3$ was 78.32 µg/L (SE = 5.79) and for AgNP was higher than the highest concentration tested (128.54 µg/L, SE = 7.13) (Fig. 2.7).

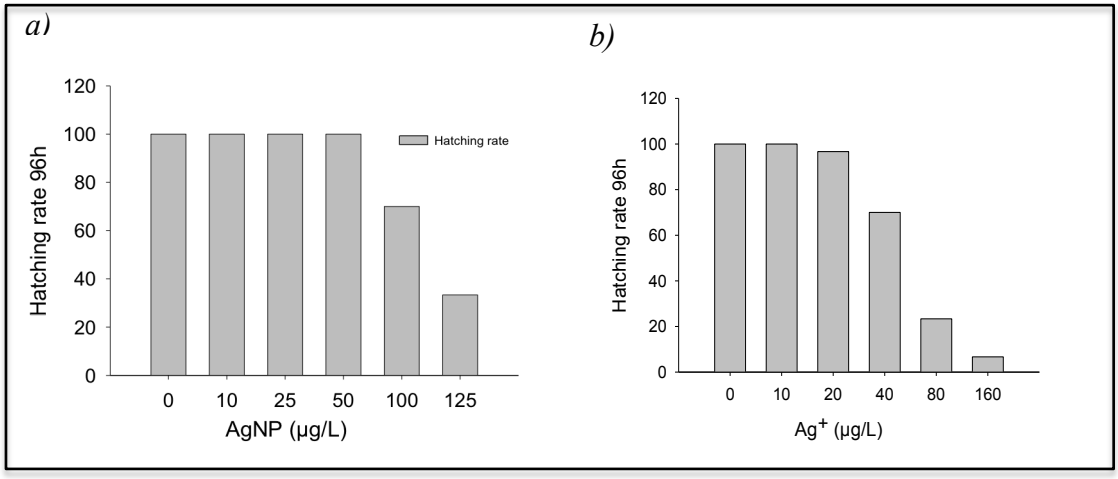

Figure 2.7. Hatching rate of of *Danio rerio* embryos exposed to A) silver nanoparticles and B) silver nitrate for 96 h. Values are expressed as total percentages.

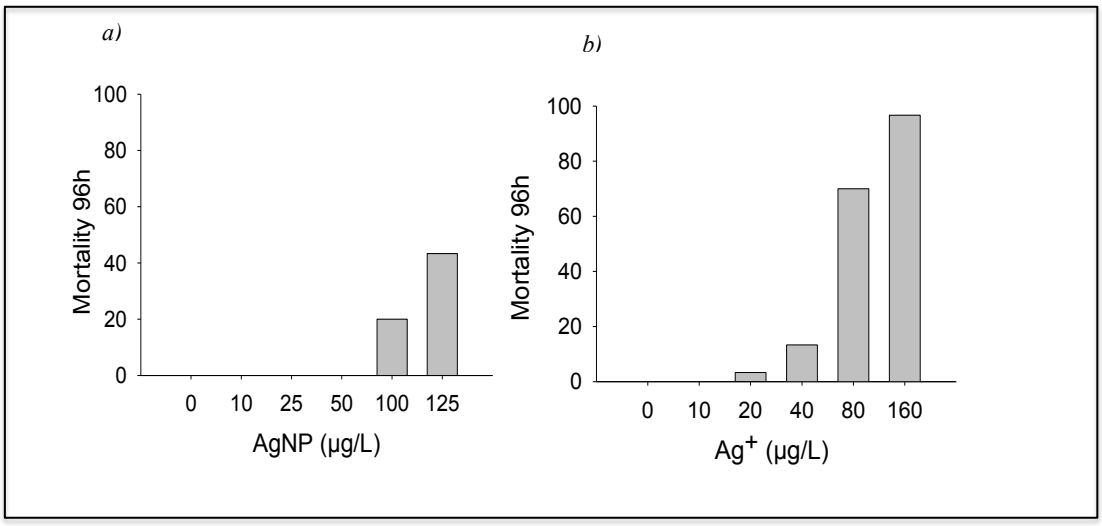

Figure 2.8. Cumulative mortality of *Danio rerio* embryos exposed to A) silver nanoparticles and B) silver nitrate for 96 h. Values are expressed as total percentages.

Despite the extrapolated value of nominal EC50, we could observe that AgNP caused harmful effects on embryo development at lower doses than the EC50. The yolk sac was not completely absorbed by 10% and 17% embryos treated with 100 µg/L and 125 µg/L of AgNP, respectively and the presence of pericardial edema was observed in one of the embryos from the concentration of 125 µg/L (Fig. 2.9 middle line). Clumps of brownish flakes were detected inside the chorionic fluid of the embryos treated with AgNP, which suggests, based on similar observations by Asharani et al. (2008), an inherent effect caused by nanoparticles as there are no similar evidences in $AgNO_3$ exposures. At 51.57 µg/L of AgNP, 50% of the embryos had these brown flakes in the chorion. $AgNO_3$ affected more *D. rerio* embryo development than AgNP, in a way that it induced a higher rate of dysmorphologies. Lower absorption of the yolk sac was observed in 20% and 7% of the embryos treated with 40 and 80 µg/L respectively of $AgNO_3$. Moreover, at 40 and 80 µg/L, 33% of the embryos showed pericardial edema and 27% of the larvae had malformation of the tail at concentrations from 20 to 80 µg/L.

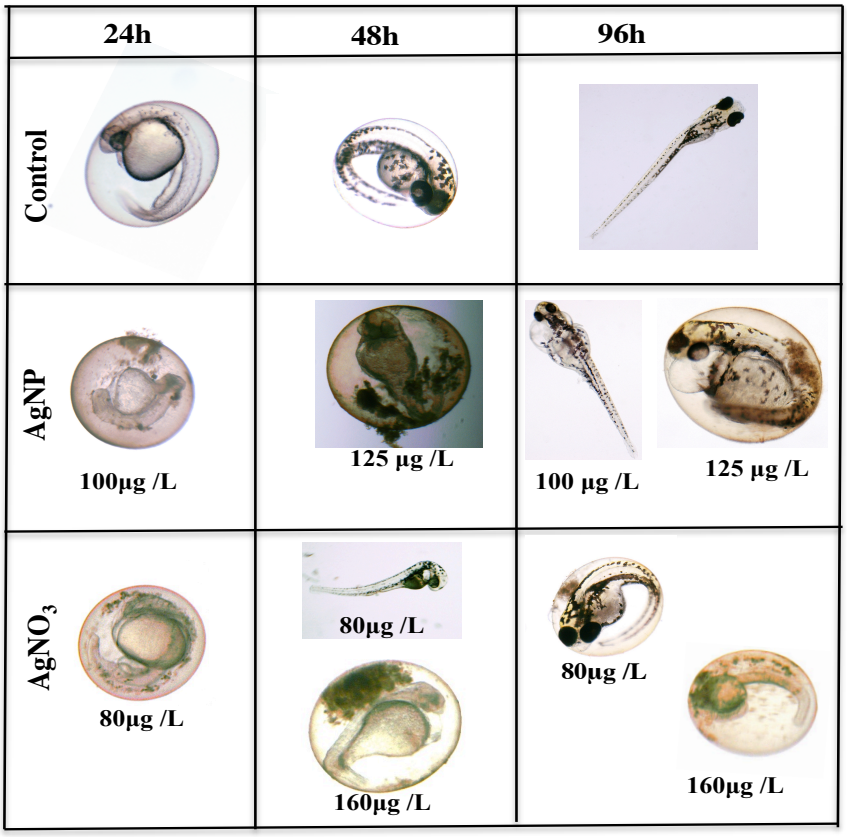

Figure 2.9. Most notable abnormalities on zebrafish embryos exposed to silver nanoparticles and silver nitrate. AgNP induced delayed hatching, noticed by the occurrence of egg stage at 96 h and formation of edema both on larvae and egg. Decreased hatching also appeared upon silver nitrate exposure. At 48 h there was an egg developmental delay compared to the control, as shown in the picture.

2.4 Discussion

One of the major concerns with respect to environmental risk assessment of nanoparticles is whether toxicity is specifically related to the nano-size of particles and consequently intrinsic properties, which differ from regular chemicals, or if toxicity is also related to the time needed for the dissolved ion to be released from the nanoparticles which would mean the risk assessment of NP's would fall into existing regulations. Several studies suggest that NPs per se are toxic to some species while other authors find no differences in toxicity between metal NPs and the corresponding dissolved ion. In this work we aimed to characterize toxicity of $AgNO_3$ and AgNP to three species that represents different levels of an aquatic trophic chain and compare their effects at relevant biological endpoints for each species, focusing on different induced effects between ionic Ag and AgNP. Our data suggests that the toxicity of both $AgNO_3$ and AgNP differs significantly based on the test species: we found no differences in toxicity for algae, a small difference for zebrafish and a major difference in toxicity for *D. magna*. Regarding the effects on growth of *R. subcapitata*, at high concentrations (100 µg/L and 125 µg/L), the percentage of growth inhibition was above 100% for both Ag forms (NP and ionic). This suggests that toxicity of both Ag forms to algae growth is dose–response dependent until 50 µg/L, and that after this concentration there is algae mortality in addition to the inhibitory growth effect. Interestingly, when we compare $AgNO_3$ and AgNP at 25 and 50 µg/L, the toxicity scenario inverts: at 25 µg/L, AgNP is twice more toxic than $AgNO_3$, whereas at 50 µg/L, AgNP is nearly twice less toxic than $AgNO_3$, which causes 100% growth inhibition. These results suggest that considering the

dynamic status of the system where algae cells are suspended in a medium that contains chloride, the higher toxicity of AgNP at 25 μg/L could be explained by the continuous release of Ag^+ ions. At the same time, these ions do not react with algae exudates and/or with the chloride in the media at the same rate as they are being released. In this way the free ions would be more readily available to the algae, causing higher toxicity than the one from the same concentration of dissolved Ag^+. In this case Ag^+ ions are reacting faster with chlorides and exudates, becoming less available. At 50 μg/L, this effect can be mitigated due to the excess of Ag and also because at higher concentrations the dissolution rates for AgNP are lower and aggregation rates are rather higher thus turning AgNP less bioavailable (Navarro et al., 2008b). Navarro et al. (2008b) compared the toxicity of $AgNO_3$ with one AgNP by evaluating the photosynthesis inhibition of *Clamydomonas reinhardtii*, and observed that based on Ag^+, AgNP was more toxic to *C. reinhardtii*. Lee et al. (2005) found that the toxicity of Ag to *R. subcapitata* was much lower in the presence of chloride when compared to its absence in the algae test media, suggesting the existence of chloride complexation and consequent decrease in bioavailable Ag^+ in the media. The mechanism of Ag toxicity to algae is related to the internalization of Ag compounds. Hiriart-Baer et al. (2006) observed that *R. subcapitata* and *C. reinhardtii* were able to accumulate more Ag as the concentration of Ag thiosulphate complexes in the medium was increased, even at low concentrations of free Ag^+. The diameter of pores across the cell wall of algae are from 5 to 20 nm, so only nanoparticles smaller than this size range are likely to be internalized (Navarro et al., 2008a). Considering the agglomeration of NPs in test-media, it is important to consider not only internalization but also the process of interaction with the cell wall as one toxic

mechanism. Under the correct conditions, nanoparticles are able to adsorb onto the external surface of the algae (Handy et al., 2012). At high particle density and consequently high aggregation rates this will impair cell division with consequent decrease on growth rate and create shading effects, which will also inhibit photosynthesis (Aruoja et al., 2008). Regarding molecular toxic mechanism, a recent study by Oukarroum et al. (2012) revealed that lipid peroxidation occurred in *Chlorella vulgaris* under exposure to AgNP which caused impairment of cell function and alterations of physic-chemical properties of the cell membrane.

We evaluate the acute toxicity of AgNP and $AgNO_3$ to *D. magna* neonates and the results presented in Table 2.1 clearly indicates that the toxicity of AgNP is highly dependent on the presence of food when compared to $AgNO_3$. That difference can be explained by the interaction between nanoparticles and the algae cells. As discussed above, when algae and AgNP are present in the media with chloride and algae exudates, the probability of the formation of NP agglomerates and aggregates is rather high. Likewise, nanoparticles once agglomerated and/or attached to the cells become less available to Daphnids reducing toxicity. On the other hand, $AgNO_3$ is readily dissolved in the media and faster available to the algae. The small difference on LC_{50} values of $AgNO_3$ in the presence or absence of food is possibly due to the greater Ag^+ toxicity to *D. magna*, and the assumption that algae in this case (if not agglomerated) can be ingested by daphnids thus increasing their fitness and tolerance. According to the Biotic Ligand Model (BLM) (Santore et al., 2001), Ag is known to compete with Na+ for the binding sites at the enzyme Na^+, K^+-ATPase, which plays an important role on the transport of Na+ and Cl^- from water to the extracellular fluid in invertebrates

(Lockwood, 1962; Péqueux, 1995). This will cause ionoregulatory failure and death of the organism (Bianchini and Wood, 2002) and it has been the most accepted mechanism to explain Ag toxicity in aquatic invertebrates such as *D. magna*. Hook and Fisher (2001) reported a LC_{50} value of 250nM based on dissolved Ag^+, for *Ceriodaphnia dubia* and Zhao and Wang (2011) found a nominal LC_{50} of 2.51 µg/L of $AgNO_3$ to *D. magna*, which is twice the LC_{50} value that we found. The higher toxicity of $AgNO_3$ compared to AgNP is likely related to the readiness release of Ag^+ in solution that will cause toxicity by ionoregulatory failure on daphnids. If the toxicity of AgNP was only driven by the dissolved Ag^+, this effect would take longer as it would depend on the dissolution of particles. The LC_{50} for AgNP found in this study is among the smallest values reported in the literature for *D. magna*. Li et al. (2010) observed a LC_{50} of 3 µg/L, while in the study of Zhao and Wang (2011) no toxicity was found up to 500 µg/L of AgNP. The differences among LC_{50} values are related to differences in particle characteristics such as capping agents, dispersants, and medium composition which will influence their behavior and dissolution in the test-media, thus their uptake and toxicity to daphnids. Sublethal endpoints for *D. magna* were also assessed in this study for the exposure to AgNP and $AgNO_3$. The feeding activity at the highest concentration of $AgNO_3$ was 50% lower than the control group. Based on the 24 h-LC_{50} for $AgNO_3$ in the presence of food, it can be assumed that $AgNO_3$ did not exert an effect on the sub-lethal endpoint such as feeding inhibition. The calculated EC_{50} for $AgNO_3$ was extrapolated, and the feeding rates were only different from the control at the highest concentration tested (which was close to the LC_{50}).

The outcome for AgNP was different from $AgNO_3$, where it was possible to

calculate a 24 h-EC_{50} for feeding inhibition. In that case, the influence of AgNP on feeding depression of *D. magna* can be assumed to be NP related, since dissolved Ag^+ induced no effects on feeding inhibition. The nanoparticle influence on feeding rates can act either due to a higher sedimentation rate of the algae to the bottom of the vessel, being this way less available to be filtered by daphnids or by accumulation of NP in their gut causing difficulty on eliminating the particles, as speculated by Zhao and Wang (2011) after exposure of *D. magna* to AgNP for 12h. The presence of clumps of nanoparticles attached to the antennae and inside the brood chamber was detected by Asghari et al. (2012) when exposing *D. magna* to low concentrations of AgNP. Indeed, if particles would adsorb onto the filtering appendage, this will decrease filtering activity and feeding rates. A long-term feeding depression is expected to negatively influence growth and reproduction. The exposure period in our study was extended to 21 days to evaluate effects of $AgNO_3$ and AgNP on reproduction and growth. Nominal values of EC_{50} calculated were 0.38 µg/L and 1.0 µg/L for $AgNO_3$ and AgNP, respectively. Under AgNP exposure, we could observe a drastically reduction on neonates production per female at 1 µg/L and 2 µg/L with the lowest EC_{50} values of AgNP for reproduction reported in the literature so far (Table 2.2). Growth was not affected by the exposure to $AgNO_3$ and AgNP. Daphnia is known to uptake NP mainly via their filtering appendages, and the transport of nanoparticles from the surrounding media to their body includes routes such as diffusion at the cell membrane, endocytosis at the cell surface and adhesion to the gut internal wall (Bhatt and Tripathi, 2011; Zhu et al., 2010). Once inside the body, nanoparticles may induce the production of reactive oxygen species (ROS), protein denaturation and DNA damage (Zhao and Wang, 2011). Regarding

dissolved Ag effects, Bianchini and Wood (2002) observed a reduction in 65% of whole body Na+ concentration in Daphnia exposed for 21 days to $AgNO_3$, which was correlated to the decrease in reproduction, also observed in this study. Until now there is no evidence of maternal transference of nanoparticles to the eggs and/or specific related effects of AgNP on egg production and deposition by Daphnia. However assumptions can be made considering physiological status of the animal affected by AgNP as an indirect effect that will negatively reflect on reproduction. As mentioned previously for the algae medium, Ag speciation in the Daphnia reproduction test was also controlled by the presence of chloride, in addition to organic matter, arising from food and from animal excretion, to which Ag can bind hence becoming less available to Daphnia, as demonstrated in previous work by Erickson et al. (1998). In order to minimize the agglomeration of NP and the formation of Ag organometalic complexes with Ag+ ions, media were renewed daily for both AgNP and $AgNO_3$ exposures (Handy et al., 2012).

FET

Zebrafish (*D. rerio*) eggs were exposed for 96 h to AgNO3 and AgNP. $AgNO_3$ had a higher toxicity to the embryos than AgNP, as indicated by the 48 h-LC_{50} values shown in Table 2.1. We could also observe developmental abnormalities of zebrafish embryos during the exposure to $AgNO_3$ and AgNP. At $AgNO_3$ exposure, the most notable effect was the delayed development and hatching of the embryos, as represented in Fig. 2.9. These effects were in agreement with the ones reported by Powers et al. (2010) who observed that $AgNO_3$ exposed embryos were smaller, showing delayed development and had small aggregates

in the chorion. Mortality occurred at concentrations from 300 μg/L and above, which are higher than the ones we applied in this work. In contrast, Asharani et al. (2008) found no toxicity of $AgNO_3$ to zebrafish embryos, with only 10% mortality at exposed concentrations of 20nM of Ag+ (equivalent to 2.17 μg/L). In our exposures, almost 80% of the embryos had agglomerates inside the chorion at 100 μg/L. Asharani et al. (2008) also observed that nanoparticles entered the eggs and showed through TEM images that particles were distributed in the cytoplasm and nucleus of cells from the trunk and tail of zebrafish larvae. Some of the embryos treated with higher concentrations of AgNP failed to properly absorb the yolk sac, thus compromising the intake of nutrients required for the normal growth and development (Bar-Ilan et al., 2009). We observed the presence of pericardial edema in hatched larvae and delayed eggs exposed to 100 μg/L of AgNP (Fig. 2.9), which was also reported by Asharani et al. (2008) and Laban et al. (2010). The appearance of edema is mainly caused by unbalanced fluid homeostasis of the body, which leads to the accumulation of fluid beneath the skin (Hill et al., 2004). The toxic mechanism of Ag ions in fish and aquatic invertebrates is related to the active transport of Ag^+ ions across the gill membrane, which will reflect on the ionoregulatory system. AgNP toxicity in this case, seems to be linked to both mechanical and chemical process, once nanoparticles are uptaken by fish through different processes from ionic Ag. Particles with different sizes, shapes and coating materials are likely to interact differently with biological molecules such as membrane proteins and DNA (Nangia and Sureshkumar, 2012). Moreover, the dissolution of nanoparticles after the mechanical interaction represents an additional source of Ag+ ions to the exposed animal, which in turn will increase toxicity of AgNP.

2.5 Conclusions

The aim of this work was to add information regarding the toxicity of AgNP compared to $AgNO_3$ to the currently "state of art" hazard assessment of nanomaterials in the environment. By comparing the data obtained in this study we have shown that the effects of AgNP in aquatic systems are closely related to the particle characteristics, the growth media and indeed the target species biology. Considering the variability of production of AgNP as well as their routes of entry into the environment it is desirable to have information on effects induced by different particles to create a robust database to regulatory bodies. Comparing toxicity effects of small AgNP to $AgNO_3$ toxicity effects, it is suggested that toxicity may be a combination of effects induced by nanoparticles and ions to organisms and which is highly dependent on the chemical composition of the media. This must be taken into account when evaluating toxicological risk of nanoparticles in natural waters, especially for freshwater algae. Although the EC50 values for AgNP and $AgNO_3$ differed for daphnids by an order of magnitude, the values measured are still considered very low, indicating that NP will be an important source for Ag exposure. Finally, the results show that the mode of action of AgNP and Ag^+ might differ since different abnormalities were obtained for zebrafish eggs and larvae. However, further investigation at the cellular and molecular level will be needed to improve the knowledge on the exact mechanism of interaction in fish.

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Chapter 3

Bioconcentration of silver nanoparticles by *Raphidocelis subcapitata*

Source: Fabianne Ribeiro

3 Uptake and elimination kinetics of silver nanoparticles and silver nitrate by *Raphidocelis subcapitata*: influence of silver behavior in solution

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Abstract

Raphidocelis subcapitata is a freshwater algae species that constitutes the basis of many aquatic trophic chains. In this study *P. subcapitata* was used as model species to investigate the kinetics of uptake and elimination of silver nanoparticles (AgNP) in comparison to silver nitrate ($AgNO_3$) with particular focus on the Ag species in solution. Algae was exposed for 48h to both AgNP and $AgNO_3$ and sampled at different time points to determine their internal Ag concentration over time. Algae media samples were collected and separated into different sized fractions: total (Ag_{tot}), dissolved (fraction below 450 nm, Ag_{dis}) and ionic (fraction below ~3 nm, Ag_{ionic}). The kinetics of uptake and elimination of Ag in algae were modelled taking the constants of decay of Ag in all the three fractions. For $AgNO_3$ exposures algae reached higher Bioconcentration Factor (BCF) values and lower elimination constants than at AgNP exposures, meaning that Ag is more readily taken up by algae in its dissolved form, rather than in its particulate form, however slowly eliminated. When the Ag_{ionic} kinetics was modelled for the AgNP exposures, a higher BCF was found. This supports our hypothesis that Ag would be internalized by algae only in its dissolved form. In addition, algae images obtained by coherent Raman scattering (CRS) microscopy demonstrated large aggregates of nanoparticles external to the algae cells with no evidences of its internalization, thus providing strong evidence that these AgNP were not able to penetrate the cells and Ag accumulation happens through the uptake of Ag ions.

Key words: *R. subcapitata, silver nanoparticles, toxicokinetics, bioconcentration factor*

3.1 Introduction

Algae play a vital role in aquatic ecosystems, due to their major function as primary producers at the bottom of the trophic chain. Consequently it is likely that any alteration of the algae community may be reflected in higher trophic levels and accordingly impact on the functioning of the ecosystem. For this reason algae is often used as a model indicator species in the risk assessment of chemicals. As the nanotechnology market expands, the production of nanomaterials and nanoparticles (NP) is rapidly increasing to supply the growing demand (Roco, 2011; Keller et al., 2013). Therefore, it is a natural assumption that nanoparticles and their transformation products will be present in the environment at some point from production and application of nanoparticle-containing products (Benn and Westerhoff, 2008; Nowack et al., 2012). The entrance of silver nanoparticles (AgNP) to the environment is predicted to commonly occur as colloidal silver, i.e. in a size range between 1 and 1000 nm and eventually result in a suspension containing metallic silver particles and Ag ions. Moreover, silver nanoparticles will likely be transformed into silver sulphide (Ag_2S) and silver chloride (AgCl) under environmental conditions (Levard et al., 2012; Z Wang et al., 2012). Today AgNP are among the most widely applied nanoparticles on the market due to their inherent antimicrobial properties. Before the advent of large-scale usage of nanotechnology, silver was already considered as one of the most toxic metals present in aquatic systems, even at the low concentrations found in natural waters. These aspects have drawn attention to the toxicity of AgNP and Ag^+ to model species in aquatic ecotoxicology. There are many ecotoxicological studies

demonstrating that AgNP induce negative effects in key species such as algae (Ribeiro et al., 2014; Oukarroum et al., 2012), zooplankton (Zhao and W-X Wang, 2010; 2011; Z Wang et al., 2012) and fish (Choi et al., 2010; Farkas et al., 2011). The interaction of Ag with algae cells depends on the size of pores across the cell wall as well as the intrinsically linked aggregation that co-insides with NP kinetics in natural systems. Both chlorides and sulphates play important roles in the mechanisms of Ag speciation; potentially reacting with Ag and creating complexes that are not reactive with algae cells (Lee et al., 2005b). Moreover, algae produce exo-polymeric substances (EPS) that are mainly composed of organic matter rich in polysaccharides. These EPS represent a dynamic source of interaction with NP in solution, causing nanoparticles to aggregate, stabilize or dissolve. EPS may also act as binding ligands to ions released from NP, thus reducing their bioavailability (Miao et al., 2009). Evidence of interactions between NP and EPS was described by (Zhang et al., 2013) who showed that amino-functionalized quantum dots (QDs) had a tendency to aggregate in the presence of EPS secreted by *Thalassiosira pseudonana*, thus becoming less likely to interact with the cells. In another study, it was observed that EPS stabilized dust derived Iron-nanoparticle (FeNP) aggregates and enhanced dissolution of Fe from the nanoparticles (Kadar et al., 2014).

Considering that several factors can influence the behaviour of nanoparticles in the media (e.g. EPS, chlorides and sulphates) we investigated how the behaviour of Ag in solution (which here will be considered as an indication of Ag speciation) would influence Ag concentration in algae. A comparison between AgNP and $AgNO_3$ exposures provides supporting information on the risk

assessment of NP to algae. Furthermore, we investigate whether AgNP are entering the cells as nanoparticles or in ionic form, by using coherent Raman scattering (CRS) microscopy. Bioconcentration is used as endpoint in this study as it relies on the chemical fractions that are available to the organism, thus it is an appropriate tool to assess nanoparticles bioavailability to algae.

3.2 Methods

Materials

Silver nitrate was purchased from Sigma-Aldrich as a crystalline powder, 99% purity CAS 7761-88-8. AgNP (3–8 nm) with an alkane coating were supplied by AMEPOX (Poland). The AgNP were dispersed in Ultra-pure water at an initial concentration of 500 mg Ag/L. The test solutions were prepared by dilution of the initial dispersion of AgNP in algae media (Woods hole MBL) to the desired concentration.

Bioconcentration tests

Algae were exposed in 500 ml Erlenmeyer's containing MBL media spiked with AgNP or $AgNO_3$. Two concentrations for both AgNP and $AgNO_3$ were tested: 15 µg/L and 30 µg/L, which correspond approximately to the EC_{10} and EC_{50}, respectively for growth inhibition of algae exposed to both silver forms (Ribeiro et al., 2014). Three replicates were performed for each concentration. The exposure phase lasted for 48h, followed by a 48h depuration phase. Test conditions were 21ºC (±1ºC) under a constant light source with replicates placed

on an orbital shaker at 70 rpm. Algae and 50 ml water samples were taken from each replicate at 0, 6, 12, 24 and 48 hours of the exposure phase to assess both the Ag concentration and the different fractions of the exposure media. Time zero sample was considered as the control. Samples were centrifuged at 2862.08*g* for 3min and 10 ml supernatant was taken to measure the total Ag concentration, here defined as the total Ag fraction (Ag_{tot}). Another 10 ml of the supernatant was then filtered through a 0.45µm polystyrene filter, which is defined here as dissolved silver concentration (Ag_{dis}). Although the term "dissolved" might also apply for the free Ag^+ in solution, we chose to use this nomenclature based on the US EPA regulation from 1995 that recommended that metals should be regulated based on the dissolved metal, as operationally defined by the fraction that is able to pass through a 0.45µm filter (US EPA, 1995).

The third fraction, corresponding to the ionic silver (Ag_{ionic}) was obtained by centrifugation of 10 ml of the supernatant at a RCF of 2862*g* for 30min using <3kDa AMICON centrifugal filters (approximate pore diameter 3 nm, Fig. 3.1). With this sample treatment, the so-called Ag ionic fraction is expected to have only ionic Ag and/or other ionic species lighter than 3KDa.

Both 0.45µm and 3kDa filters were pre-treated with a solution of 0.1M $Cu(NO_3)_2$ to avoid losses of Ag to the filter (Cornelis, Kirby, Beak, Chittleborough, & McLaughlin, 2010). To measure Ag elimination from the algae, the remaining algae in the Erlenmeyer's were centrifuged, washed 3 times with Milli-Q water and re-suspended in freshly prepared, un-dosed MBL media for the 48h depuration phase.

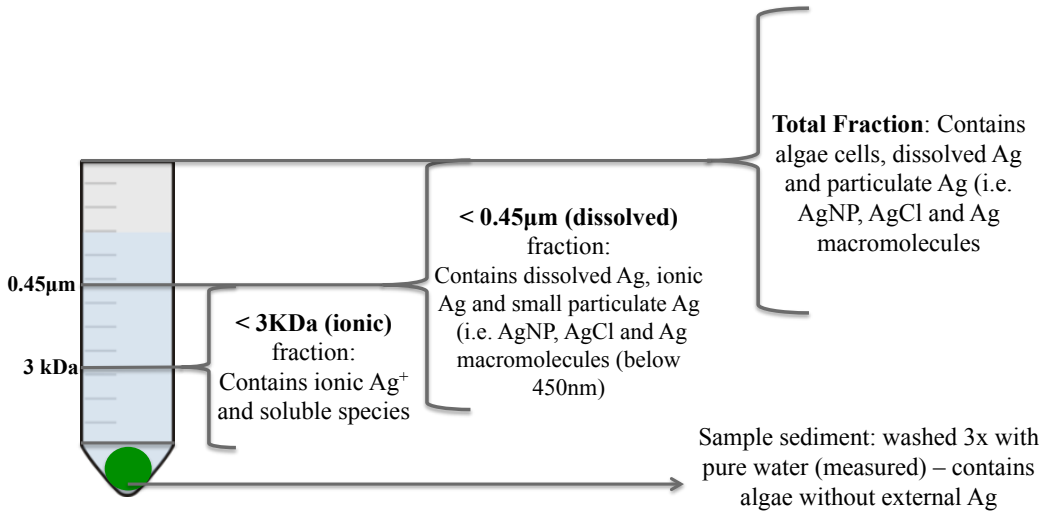

Figure 3.1. Schematic representation of silver fractionation in AgNP and $AgNO_3$ exposure media. See methods section for better description of each Ag fraction.

Particle characterization

Aggregation experiment on algae media was performed by a Malvern Zetasizer equipment and Zetasizer software 6.20. Short and long term experiments were conducted. For the short (minutes) experiments, the initial stock nanoparticles suspension was diluted with the media to the desired concentrations in normal DLS cuvettes and inserted immediately in the instrument. The measurement was started at a fixed attenuator and measurement position to avoid the optimization time, the correlation time was set to 2 s and 120 data points were generally obtained. For the long-term experiments (days), the first measurement (day zero) was obtained by creating an average result from the short-term data points. The cuvettes were stored in the dark and three measurements were performed (3 runs of 20 s each) in the following days. To evaluate the effect of particles sedimentation, the samples were shaken after performing the measurement and a new measurement was done. Derived count rates are

included in the long-term experiments to compare the capacity of the remaining particles (large and small) to scatter light.

For TEM imaging, an initial suspension of AgNP (1000 mg/L) was diluted to 100 mg/L in MBL media and a drop of this suspension was deposited onto a holey carbon coated Cu TEM grid and dried at room temperature for several hours before examination. Experiments were carried out on a JEOL 2010 analytical TEM with a resolution of 0.19 nm, an electron probe size down to 0.5 nm and a maximum specimen tilt of ±10° along both axes. The instrument is equipped with an Oxford Instruments LZ5 windowless energy dispersive X-ray spectrometer (EDS) controlled by INCA.

Sample digestion

Water

Prior to digestion, all water samples (10 ml) were mixed with 0.28 ml of H_2O_2 and 1.35 ml of HCl, bringing the concentrations to 1% of H_2O_2 and 5% of HCl for 24h. This procedure aimed to break down organic matter in the samples and ensure that any silver adsorbed to the sample holder´s wall was released as soluble AgClx complexes.

After 24h samples were transferred to Teflon beakers (25 ml volume capacity) and allowed to evaporate on a hotplate over 45-50ºC (without boiling) until 1-1.5 ml of the sample remained in the beakers. Samples were then mixed with 1 ml HNO_3 (65% trace analysis) and 3 ml of HCl (37% trace analysis) before being heated for 1 hour. All samples were transferred to plastic graduated tubes and diluted with a 1% HCl to a final volume of 45 ml. 3 replicates of un-dosed MBL

media and 3 replicates of a known concentration of Ag were digested together with all other samples to be used as blank controls and as reference material for Ag recovery respectively. Total silver was measured by Graphite Furnace Atomic Absorption Spectroscopy (GF-AAS).

Tissue

The algae pellet obtained after centrifugation was dried at 50ºC, weighed and transferred to Teflon beakers for digestion. 3 ml of HNO_3 (65% trace analysis) were added to the beaker (with lids on) and heated on a hotplate at 50ºC for approximately 30 min (or until the tissue dissolved). Samples were allowed to cool down at room temperature and mixed with 1ml of HCl (37% trace analysis) before being replaced on the hotplate with the lids on and heated for another 30 min. After 30 min., the lids were removed and samples were allowed to evaporate (without boiling) until approximately 1ml remained. In addition, 3 replicates of the reference material DOLT-4 (Dogfish Liver Certified Reference Material for Trace Metals) were digested with the other tissue samples. The dilution and Ag measurement steps followed the same procedure described for water samples.

Toxicokinetic modeling

In the present study a one-compartment model was used to describe the kinetics of the bioaccumulation of Ag from AgNP and $AgNO_3$ in *R. subcapitata*. This model describes the fate of Ag based on mass balance equations. Assuming that the concentration of exposure is constant, two equations were used to model uptake and elimination of AgNP and $AgNO_3$ in the algae:

$$Q(t) = \frac{k1}{k2} \times Cexp \times (1 - e^{(-k2xt)}) \quad Equation_1 \text{ / } \textbf{uptake}$$

$$Q(t) = \frac{k1}{k2} \times Cexp \times (1 - e^{(-k2*(t-tc))} - e(^{-k2\times t)}) \quad Equation_2 \text{/ } \textbf{elimination}$$

Where:

Q(t) is the concentration in the algae at time t

tc is the time (hours) at which algae were transferred to uncontaminated media

k_1 is the uptake rate constant (L/g/hour)

k_2 is the elimination rate constant (1/hour)

C*exp* is the concentration of exposure

e stands for the exponential function

The Bioconcentration Factor (BCF) was calculated by the ratio of k_1 and k_2. Considering the decrease in concentration of silver in the media during exposure, a decay rate constant (k_{dec}) was modelled by fitting the following equation to the concentrations of Ag measured at different points in time:

$$Ag(t) = [Ag] \times e^{(-kdec \times time)}$$

This decay constant was included in the uptake model (Equation 1) for different fractions to read:

$$Q(t) = \frac{k1}{k2-kdec} \times Cexp \times e(^{-kdec\times t}) - e(^{-k2\times t}) \quad Equation\ 3$$

Where:

Q(*t*) is the concentration in the algae at time *t*

k*dec* is the Ag concentration decay rate constant (1/hour)

Coherent Raman scattering (CRS) spectroscopy.

In order to investigate whether Ag was uptaken by algae in its nanoparticle form or in the ionic form (Ag^+), different treatments were setup to be imaged by CRS spectroscopy. For sample preparation, suspended algae in AgNP media, previously exposed for 12 hours was sampled and *1)* centrifuged at 2862.08g for 3 minutes, then washed 3 times with milli-Q water to remove weakly adsorbed AgNP from the external cell wall, and fixed in a single-strength glutaraldehyde fixative (4%) in cacodylate buffer at room temperature for 4 hours; *2)* centrifuged at 2862.08g for 3 minutes and proceeded to fixation; *3)* suspended cells were directly placed in the fixative (without centrifugation nor washing). After the fixation period, algae were then placed on a microscope slide with a cover slip.

CRS theory

CRS microscopy is a novel microscopy technique that provides label-free contrast, based on vibrational spectroscopy (Moger, Johnston, & Tyler, 2008) which has exceptional capability for locating metal nanoparticles particles within biological tissues with subcellular precision (Johnston et al., 2010). CRS microscopy derives its contrast from intrinsic molecular vibrations in a sample.

The CRS process involves two lasers where the frequency of the first laser is constant, while the frequency of the second one can be tuned in a way that the frequency difference between the two lasers equals the frequency of the Raman-active or vibrational mode of interest. CRS imaging was performed using a custom- built microscopy system based on a commercial confocal laser- scanning microscope and a synchronized dual-wavelength picosecond laser source. Laser excitation was provided by an optical parametric oscillator (OPO) (Levante Emerald, APE, Berlin) pumped with a frequency doubled Nd:vandium picosecond oscillator (High-Q Laser Production GmbH). The pump laser generated a 6 ps, 76 MHz pulse train at 532 nm with adjustable output power up to 10 W. The OPO produced collinear signal and idler beams with perfect temporal overlap and provided continuous tuning over a range of wavelengths. The signal beam was used as the pump, ranging from 670 to 980 nm and fundamental of Nd:vandium (1064 nm) used as the Stokes beam. The maximum combined output power of the pump and Stokes was approximately 1 W, which was attenuated to reduce the power at the sample to between 15 and 30 mW. To improve the transmission of the near-IR excitation through the commercial microscope (IX71 and FV300, Olympus UK) the galvanometer mirrors were replaced with silver mirrors and the tube lens was replaced with a MgF2 coated lens. The collinear pump and Stokes beams were directed onto the scanning confocal dichroic which was replaced by a silver mirror with high reflectivity throughout the visible and NIR (21010, Chroma Technologies, Bellows Falls, VT). The forward-CRS signal was collected by the air condenser, transmitted by the dichroic mirror and directed onto a red-sensitive photomultiplier tube (R3896, Hamamatsu Photonic UK). The epi-CRS signal was collected using the objective

lens and separated from the pump and Stokes beams by a long-wave pass dichroic mirror (z850rdc-xr, Chroma Technologies) and directed onto a second R3896 photo- multiplier tube at the rear microscope port. The CRS signal was isolated at each photodetector using a single band-pass filters centered at the anti-Stokes wavelengths. Imaging was performed using either a 60 × water immersion, or 20 × air objective (UPlanS Apo, Olympus UK)

3.4 Results

Particle characterization

The hydrodynamic diameter of the AgNP in water measured at 127–132 ±4 nm was shown to be reasonably stable for the duration of the experiment (3.2). In MBL at day zero, the average size of agglomerates was approximately 100 nm and after three days the agglomerate size varied between 200 and 250 nm (Fig. 3.2A)

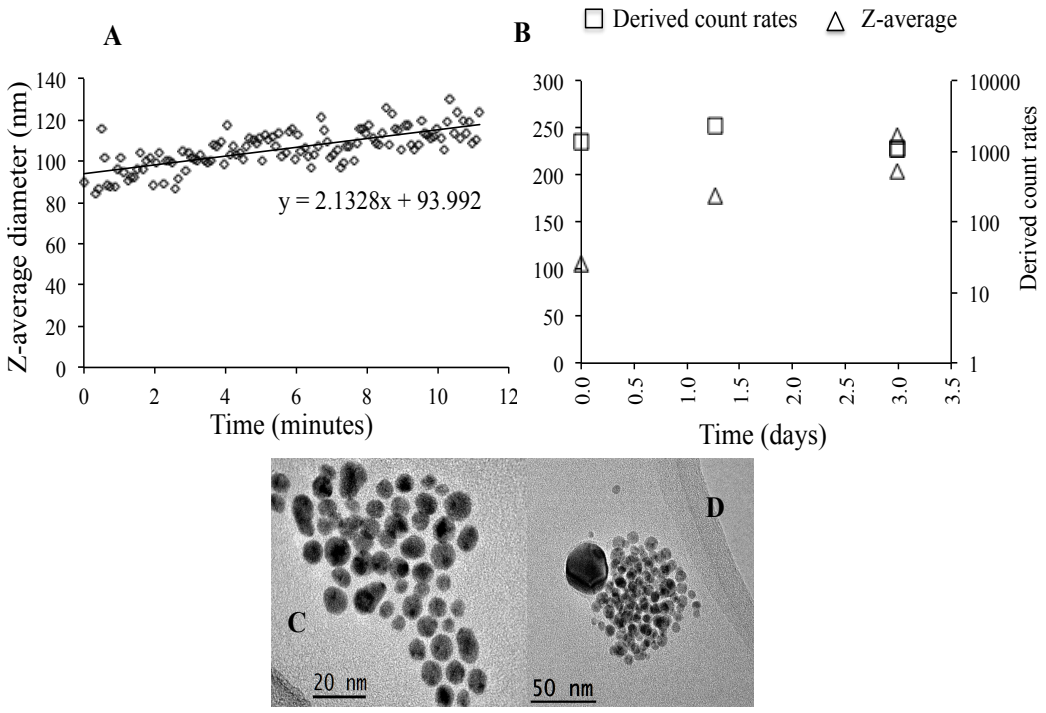

Figure 3.2. (A) Z-average hydrodynamic diameter (nm) of silver nanoparticles in MBL media at 1 mg\L (short-term experiment) (B) Z-average hydrodynamic diameter (nm) and derived count rates of silver nanoparticles in MBL media at 1 mg\L (long-term experiment)(C)- TEM image of initial suspension of AgNP in milli-Q water. (D)- TEM image of AgNP in MBL media.

Ag fractionation in the exposure media

Ag concentration in the $AgNO_3$-spiked media decreased until 6h of exposure, regardless the concentration and the fraction (Ag_{tot} and Ag_{dis}). For this exposure, all fractions had a pronounced decrease in concentration until 6h, after which concentrations remained reasonably constant (Fig. 3.3*a*).

As expected, ionic concentrations were higher in the media containing $AgNO_3$, confirming that silver nitrate is more readily available in the soluble form (Ag^+) than Ag^+ released from AgNP. However, the pattern of decay of the concentration of ionic Ag arising from $AgNO_3$ was different for different concentrations: at 15

μg/L, ionic Ag concentration decreased rapidly over the first 6h of exposure, after which it slightly increased and remained constant until the end of the exposure, while at 30 μg/L, concentration decrease continued until 12h of exposure, afterwards following the same pattern as at 15 μg/L (Fig. 3.3*c*).

In the media containing AgNP, Ag concentration decreased for both concentrations and fractions (Ag_{tot} and Ag_{dis}) until 24h of exposure, after which there seemed to be an increase in concentration again, except for the dissolved fraction at 30 μg/L, which remained constant (Fig. 3.3*b*). The ionic Ag concentrations in the AgNP exposure media showed a rapid decrease from zero to 6 hours for both 15 μg/L and 30 μg/L (Fig. 3.3*d*). From 6h to 24h, the ionic Ag concentration continued to decrease slowly at 30 μg/L, whilst at 15 μg/L, it appeared to remain constant. At both exposure concentrations, ionic Ag concentration showed a slight increase after 24h.

Constants of Ag concentration decay in solution are presented in table 3.1, separate for each fraction. For AgNP exposure, the bigger the fraction, the lower the decay rate constant was, with Ag_{dis} at 15 μg/L being the fraction with the highest decay rate constant. For $AgNO_3$, the Ag concentration decay rate constants in the exposure media were much higher than for AgNP. For both the Ag_{tot} and Ag_{dis} fractions, concentration decay rate constants increased with increasing concentration. On the other hand, for Ag_{ionic}, a reverse pattern could be observed, with the highest decay rate constant being found at 15 μg/L compared to 30 μg/L. Furthermore, after 24h of exposure, Ag_{ionic} from AgNP and $AgNO_3$ reached very similar concentrations, which may indicate that dissolution is not only concentration-dependent but also time-limited.

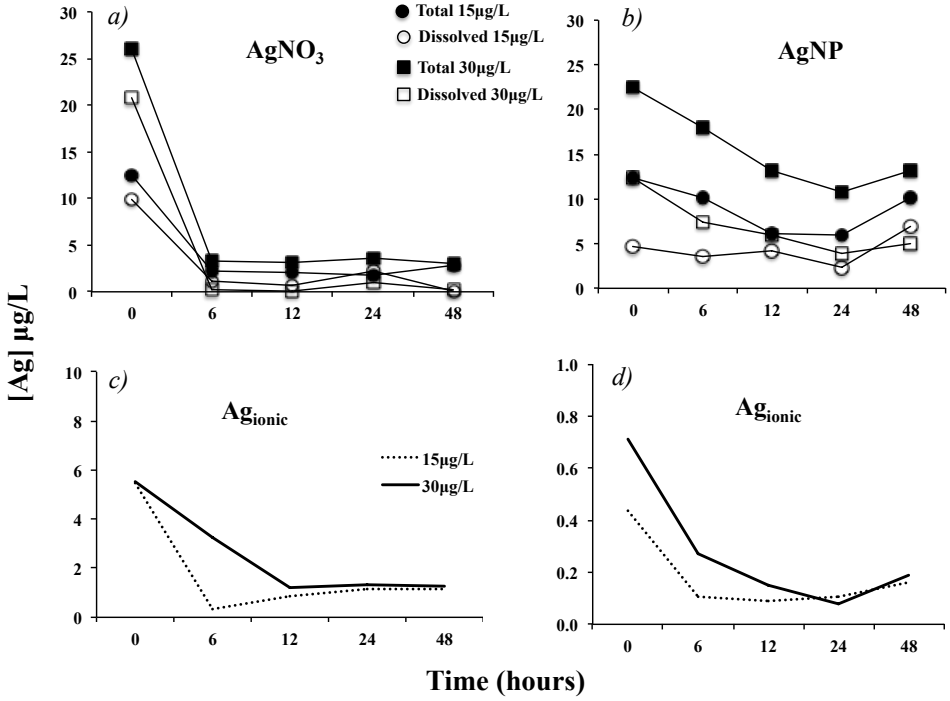

Figure 3.3. Total, dissolved (*a* and *b*) and ionic (*c* and *d*) silver concentrations in each sampling time (hours) for AgNP and $AgNO_3$ in MBL media. Please note the 10-fold difference on the X-axis between figures *c* and *d*.

Regarding the media containing AgNP, we could observe a decrease in Ag concentration for both concentrations and fractions (Ag_{tot} and Ag_{dis}) until the 24h of exposure, after which there seems to be an increase in concentration again, except for the dissolved fraction of 30 µg/L, which remains constant (Fig. 3.3*b*). Regarding the ionic Ag concentrations on AgNP exposure media, the pattern of rapidly decrease in concentration from zero to 6 hours was again observed for both 15 µg/L and 30 µg/L (Fig. 3.3*d*). From 6h to 24h, concentration of ionic Ag at 30 µg/L continues to decrease in a slowly rate,

whilst at 15 µg/L, the ionic Ag concentration appeared to remain constant. At both exposure concentrations, ionic Ag had a slightly increase after 24h.

Constants of Ag decay in solution are present in table 3.1 and presented according to each fraction. For AgNP exposure we could observe that the bigger the fraction, the lower is the decay constant, being the Ag_{dis} of 15 µg/L, the fraction with a higher decay constant. Regarding the $AgNO_3$ exposure media, the decay constants of Ag in solution are much higher than those calculated for AgNP. For both Ag_{tot} and Ag_{dis} fractions, constants are increased with increasing concentration. On the other hand, when Ag_{ionic} is considered, a reverse pattern can be observed, where the higher decay constant was calculated for the concentration of 15µg/L compared to 30µg/L. Furthermore, at 24h of exposure, Ag_{ionic} from AgNP and $AgNO_3$ reached very similar concentrations, which may indicate that dissolution is not only concentration-dependent but also time-limited.

Toxicokinetics and Ag internalization

Uptake and elimination kinetics of *R. subcapitata* were modelled according to the one-compartment model, in which the rate constants of uptake from water (k_1) and elimination rate constants (k_2) were calculated for exposures to AgNP and $AgNO_3$. Assuming that Ag concentration remained constant during the uptake phase, the algae accumulated roughly 50 µg/g (dw) of Ag when exposed to AgNP, in comparison to ~100 µg/g (dw) upon exposure to $AgNO_3$ at the EC_{10} concentration (Fig. 3.4 and 3.5). In the $AgNO_3$ exposure media, the maximum Ag

burden in algae (average) reached 140 µg/g (dw) (SE=16.6) at 15 µg/L after 12 h whereas at 30 µg/L the maximum Ag burden after 12h was 342 µg/g (dw) (SE=40.9). For AgNP the Ag burden in algae reached 45.0 µg/g (dw) (SE=1.10) after 24h of exposure to 15 µg/L and 93.7 µg/g (dw) (SE=8.49) after 24h of exposure to 30 µg/L. When related to nominal Ag concentrations, uptake and elimination rate constants were much lower in the AgNP treatments compared to $AgNO_3$ (table 3.2) indicating that kinetics was faster when the algae were exposed to $AgNO_3$ that to AgNP.

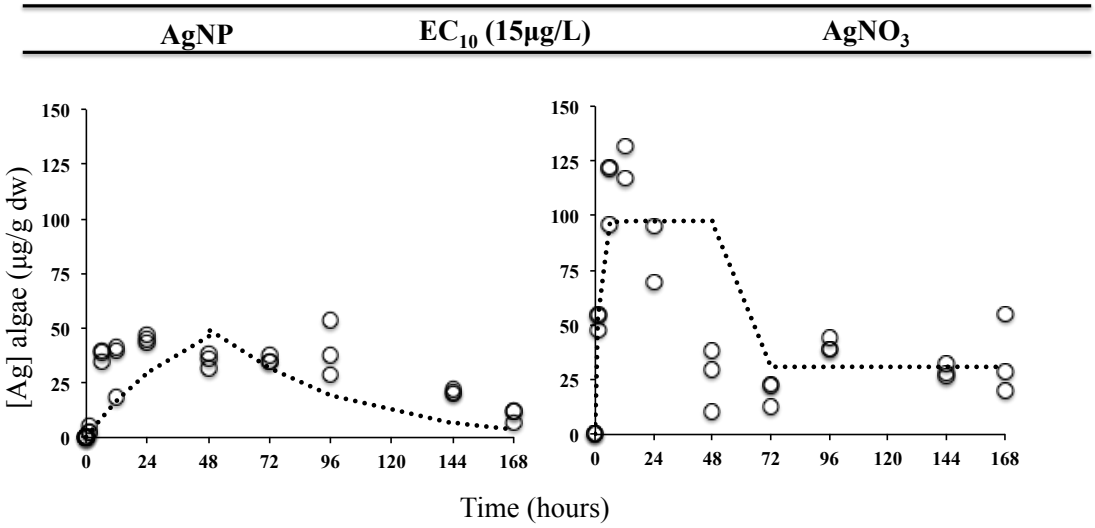

Figure 3.4. Uptake and elimination kinetics of silver in *Raphidocelis subcapitata* exposed to 15µg/L of AgNP and $AgNO_3$. The kinetic model curve was calculated by equation 1 for the uptake phase and equation 2 for the elimination phase, using algae body burdens. The elimination phase started at 48h.

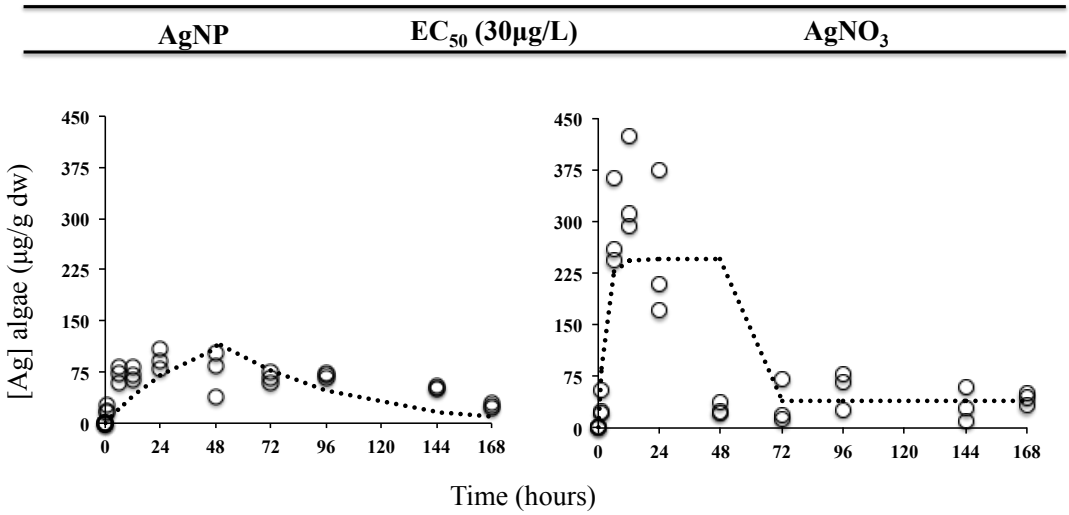

Figure 3.5. Uptake and elimination kinetics of silver in *Raphidocelis subcapitata* exposed to 30µg/L of AgNP and $AgNO_3$. The kinetic model curve was calculated by equation 1 for the uptake phase and equation 2 for the elimination phase, using algae body burdens. The elimination phase started at 48h.

Table 2.1. Decay constants (µg/L/hour) of Ag in MBL media, according to the fraction of AgNP and $AgNO_3$ along a 48h of exposure experimental setup.

	AgNP			**$AgNO_3$**		
	Ag_{tot}	Ag_{dis}	Ag_{ionic}	Ag_{tot}	Ag_{dis}	Ag_{ionic}
15µg/L	0.01	0.03	0.18	0.24	0.34	0.42
30µg/L	0.02	0.04	0.14	0.31	0.72	0.30

When Ag concentration decay rate constants were included in the model, the pattern observed for nominal concentrations remained; i.e., for all fractions (Ag_{tot}., Ag_{dis}., and Ag_{ionic}) BCF values were higher for $AgNO_3$ than for AgNP exposure (table 3.2). Strictly focusing on BCF values calculated based on the dissolved fraction (Ag_{dis}), the difference between AgNP and $AgNO_3$ was the

lowest compared to other fractions, meaning that concentration of Ag in algae was mainly attributed to the ionic form of silver. This was confirmed by the CRS imaging (Fig. 3.6) where it was clearly seen that the AgNP agglomerated externally to the cell. Reconstructing multiple series of images separated by 0.25μm in the z-plane into a three dimensional image provided no evidence of AgNP internalization in any preparation group. The AgNP changed in size according to the sample preparation method, with unwashed and uncentrifuged preparations displaying larger visible aggregates outside the cell.

3.5 Discussion

This work focused on the study of the mechanisms underlying the bioconcentration of Ag in a freshwater algae species *Pseudokirchneriella subcapitata* by comparing exposures to AgNP and to $AgNO_3$. In order to better understand how the chemical reactivity of Ag with other constituents of the media and test conditions could influence uptake, the toxicokinetics were modelled by taking into account different sizes of Ag complexes, from large particle agglomerates/aggregates to the dissolved and ionic species. From the fractionation analysis of AgNP-spiked exposure media, it was found that the Ag_{tot} and Ag_{dis} fractions at both the EC_{10} and EC_{50} levels (15 and 30 μg/L, respectively), decreased up to 24h of exposure after which concentrations started to increase again until the experiment was terminated after 48h. In $AgNO_3$ spiked media, however, the Ag_{tot} and Ag_{dis} fractions decreased rapidly during the first 6 hours of exposure, and remained at low levels until 48 hours.

Moreover, during the period of 0 to 12 hours, Ag body burdens in algae increased exponentially as shown in figures 4 and 5. It is therefore likely that the decrease in Ag concentration in the media was due to the fact that algae were taking up Ag from solution. Nonetheless, considering the system dynamics, the decrease in the dissolved Ag (Ag_{dis}) concentrations was more pronounced in the $AgNO_3$ treatment than for AgNP, reaching 2 µg/L after 6h. As Ag_{dis} contained dissolved Ag, Ag complexes and Ag bound to macromolecules (such as EPS), the rapid decrease in Ag_{dis} concentration in the $AgNO_3$ treatment is likely to be associated with its bioavailability to algae. This was not observed for the AgNP exposure media due to the presence of small particles (<450 nm) in the Ag_{dis} fraction, that could have been internalized by the algae.

As demonstrated by (Lee et al., 2004), the uptake of Ag by *P. subcapitata* was not influenced by the concentration of chloride in the media, meaning that algae can readily internalize AgCl complexes.

Dissolution or release of ionic silver (Ag^+) was also measured over time for both algae exposures to AgNP and $AgNO_3$. Dissolution was observed to occur faster for the $AgNO_3$ exposure compared to AgNP, as shown in figure 3. For both exposure treatments at 15 µg/L, the concentration of ionic silver decreased rapidly in the first 6h before levelling off. However, at 30 µg/L, released Ag^+ from AgNP and $AgNO_3$ continued to decrease in concentration until 12h and 24h of exposure, respectively. This is opposite to the trend observed by (Lee et al., 2005a) who reported that the concentration of ionic Ag released from AgNP increased exponentially on during the first 6h of exposure. However, that study was performed in deionized water, whilst our experiment used a culture media in the presence of algae. Considering that the internal concentration of Ag in *P.*

subcapitata increased for both treatments during the first hours of exposure (fig 4 & 5), it is likely/possible that algae were readily taking up most of the released Ag^{+}, therefore leading to the decrease in Ag concentrations in the test media.

The decay of ionic Ag concentrations correlated with the maximum Ag concentration in the algae in the $AgNO_3$ exposure media where the concentration of Ag_{ionic} decreased with time until the 6th hour of exposure to 15 µg/L and while a maximum in the algae body burden was reached after 12h of exposure. This may indicate a transfer of Ag_{ionic} from the media to the algae. Moreover, algae dosed with $AgNO_3$ seemed to have a faster uptake of Ag during the first hours of exposure and started to eliminate Ag already during the uptake phase. This indicates that no uptake/elimination equilibrium was reached, and that Ag was continually being internalized and eliminated by the algae at varying rates before they were transferred to Ag-free media.

Table 3.2. Kinetic parameters estimate by equation 3 for each fraction of AgNP and $AgNO_3$. Ag_{nom} was calculated based on nominal concentrations, Ag_{tot} was based on the total fraction, Ag_{dis} was obtained by the 0.45µm-filtered fraction (dissolved) and Ag_{ionic} was based on the ionic Ag obtained by ultracentrifugation with 3KDa membranes. K_1 – uptake rate constant (L/g/hour); K_2 – elimination rate constant (hour); BCF – bioconcentration factor.

		15µg/L				30µg/L			
		Ag_{nom}	Ag_{tot}	Ag_{dis}	Ag_{ionic}	Ag_{nom}	Ag_{tot}	Ag_{dis}	Ag_{ionic}
AgNP	K_1	0.11	0.51	0.42	0.58	0.13	0.39	0.41	0.53
	K_2	0.02	0.13	0.07	0.01	0.02	0.07	0.05	0.01
	BCF	4.50	3.86	5.73	74.98	5.44	5.68	7.65	45.94
$AgNO_3$	K_1	5.28	3.02	3.76	4.40	3.51	4.32	7.87	4.79
	K_2	0.81	0.03	0.03	0.02	0.43	0.03	0.02	0.03
	BCF	6.51	100	125	220	8.16	144	393	159

Assuming that Ag uptake followed a first-order kinetics model and considering that the algae cells behaved as one compartment, we were able to calculate uptake (k_1) and elimination (k_2) rate constants and bioconcentration factors (BCF) for the different Ag fractions. These parameters, presented in table 3.2, will be hereafter used to guide our interpretation of the Ag toxicokinetics in algae. The uptake rate constants related to all Ag fractions remained higher in the $AgNO_3$ exposures, while the elimination constants based on Ag_{tot} and Ag_{dis} were lower for $AgNO_3$. It has been demonstrated that the mechanism behind Ag internalization in algae is related to accidental cation transport, which is believed to occur through the same mechanism as the internalization of essential cations (i.e, Na^+, K^+). This is due to the inability of the system to distinguish between Cu^+ and Ag^+, given that both metals share a few chemical characteristics (Solioz and Odermatt, 1995; Lee et al., 2004). Additionally, $AgCl^0$ is likely to be internalized through passive diffusion from the external cell environment to the cytosol, over protein channels in membranes (Lee et al., 2004). Either

mechanism could explain the higher k_1 values obtained for $AgNO_3$ exposures as, in this case, Ag is more likely to be present as Ag^+ and consequently in a form more easily internalized by the algae. On the other hand, lower elimination constants indicate that the algae failed to completely excrete Ag either as Ag^+ or Ag complexes. Ag is known to be associated with metallothioneins (MT) (Robinson, 1989) thus it is likely that ionic Ag in algae is sequestrated and bound or stored in such a way that it cannot be eliminated anymore, or it is eliminated in a rather slow rate. Such binding to MT is less likely for non-ionic Ag of or Ag complexes or particles.

The BCF of AgNP was the highest when we considered the ionic fraction, which corroborates the hypothesis that algae would uptake dissolved Ag and/or Ag complexes from the media rather than Ag particulate forms. Moreover, the highest BCF value was obtained at the lowest AgNP concentration (15 µg/L) indicating that dissolution rates of AgNP were more efficient at lower concentrations and consequently at less aggregation rate of particles probably driven by a steeper concentration gradient. This was also reported by (Kittler et al., 2010) who observed that PVP coated AgNP showed a higher dissolution at 0.05 g/L when compared to 0.1 g/L, after approximately 100 days in a long-term dissolution experiment.

The lower BCF values obtained when using the total and dissolved fractions from AgNP were interpreted as an indication of the behavior of AgNP in the exposure media. As mentioned before, algae segregate exudates that are mainly composed of organic matter, rich in polysaccharide molecules, which in contact with AgNP can induce aggregation of nanoparticles thus altering their bioavailability to algae (Joshi et al., 2012). Our results suggest that in this instance these

exopolymeric substances produced by algae played a role in decreasing the bioavailability of AgNP to the cells.

By using the CRS spectroscopy imaging technique, *P. subcapitata* exposed to AgNP showed no evidence of internalization of the nanoparticles used in this experimental setup. Aggregates varying in size could be visualised externally associated with the cell wall, but no particles were detected internally after 3D image sectioning of the cells, which confirms that all AgNP signals originated from outside the cell. This suggests that all internalized Ag was in the form of dissolved Ag or $AgCl_x$ complexes. However, the nanoparticles agglomerates nearby algae cells may induce a physical effect on algae, depending on the size of agglomerates and may interfere with the algae growth and/or lead to a faster sedimentation of algae. In addition, shading effects could also play a role in toxicity, depending on the ability of the nanoparticle to attach to the cell surface. NP may thus interfere with photosynthesis and/or by their chemical characteristics the nanoparticles may induce the formation of a turbid media which can suppress the light absorbance by algae (Aruoja et al., 2009; Schwab et al., 2011). Thus, our data highlight the importance, when predicting the potential risk of nanoparticle presence in the environment, of taking into consideration both the chemical and physical effects of nanoparticles interacting with phytoplankton.

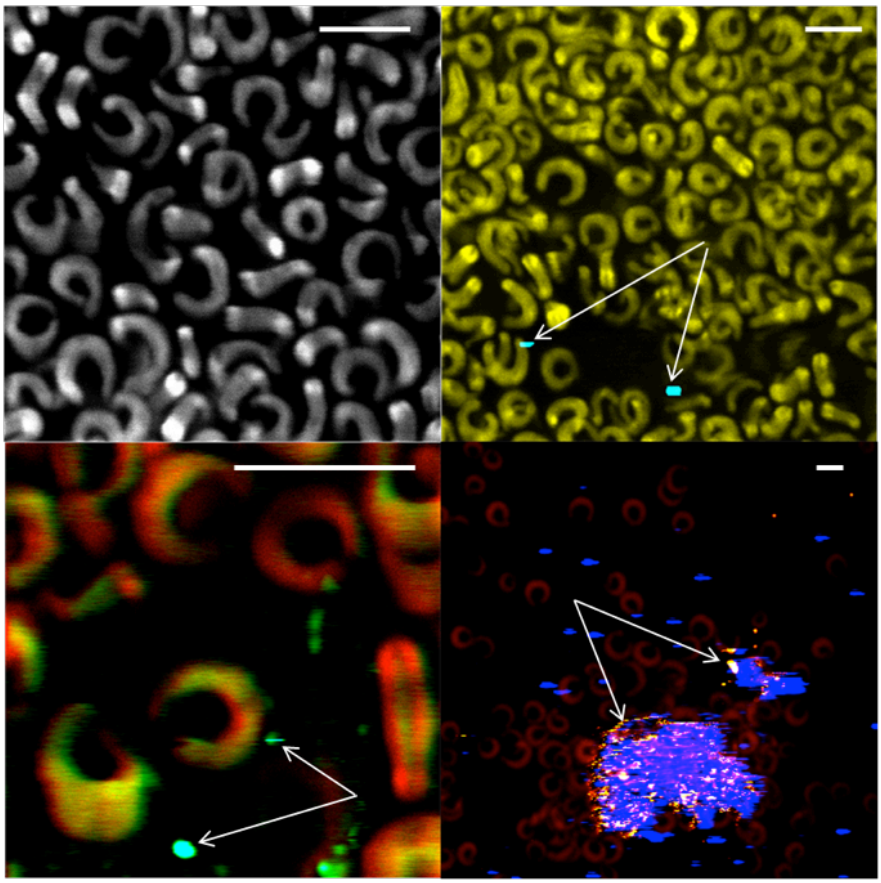

Figure 3.6. CRS images of *Raphidocelis subcapitata*. (A) control. (B, C) dosed with 15 µg AgNP /L: C centrifuged and washed with Milli-Q water. (D) dosed with 15 µg AgNP /L centrifuged and unwashed. Arrows indicate nanoparticle signal. Figure B and C show small agglomerates outside and not attached to the cell surface, and on figure D large NP agglomerates can be seen outside cells. Scale bars are 10 µm.

3.6 Conclusions

In conclusion, we have demonstrated that the separation into different size fractions revealed to be a trustworthy tool to study silver chemical behavior in our test media (MBL) and helped to estimate the relationship between silver speciation and its bioavailability to algae. *R. subcapitata* was able to concentrate silver inside the cells when they were exposed to both AgNP and $AgNO_3$ at 15 and 30 µg/L. The amount of silver taken up by the algae was dependent on the uptake and elimination rate constants, which in the $AgNO_3$ exposures resulted in higher BCF values. When BCF was calculated on the basis of dissolved Ag from AgNP, a higher value was obtained in comparison to the other Ag-sized fractions, indicating that upon AgNP exposure silver was internalized as Ag^+ or $AgCl_x$ complexes rather than in its particulate form. Finally, CRS images showed that AgNP used in this study were unable to cross algae cell walls, which lead us to conclude that bioconcentration of AgNP is probably mediated by the internalization of Ag ions.

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3.7 Supplementary material

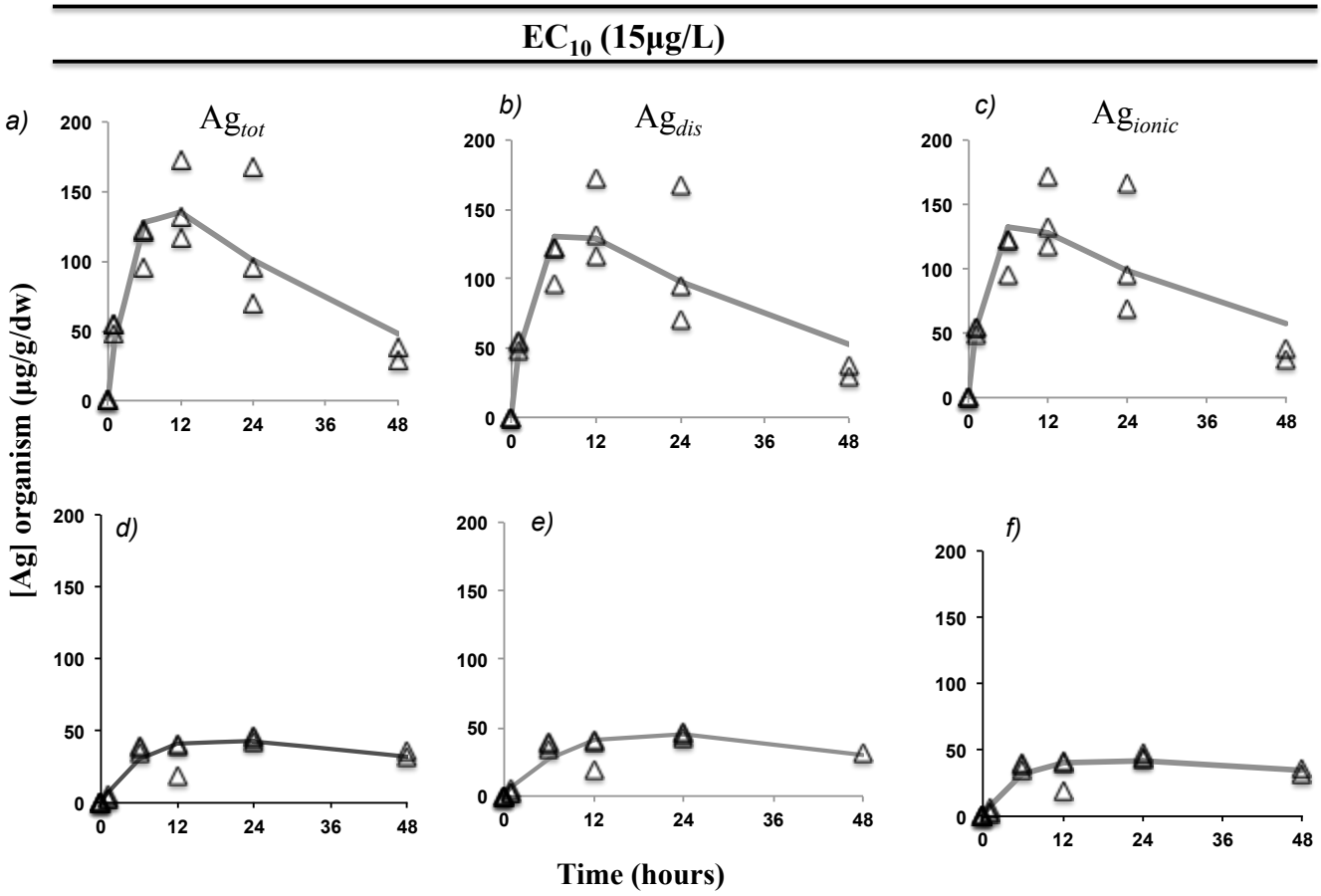

Figure 1. Uptake kinetics of silver modeled based on algae burdens considering each silver sized-fractions at the EC_{10} level (15µg/L) for silver nitrate (upper graphs) and silver nanoparticles (bottom graphs) exposures. Ag_{tot}- total silver; Ag_{dis}- dissolved silver; Ag_{ionic}- ionic silver; for more details on the fractions please see the Material and Methods section.

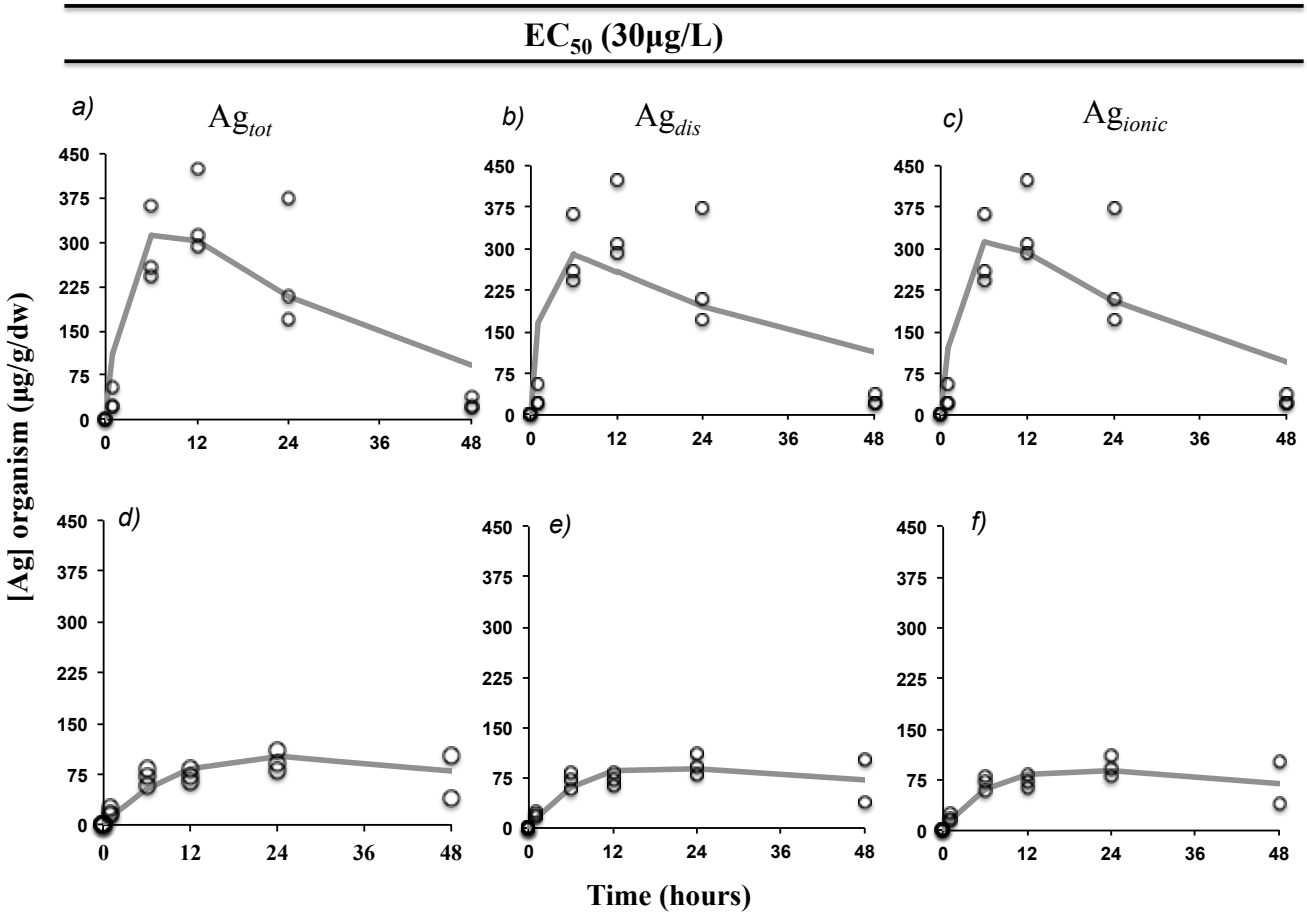

Figure 2. Uptake kinetics of silver modeled based on algae burdens considering each silver sized-fractions at the EC_{50} level (30µg/L) for silver nitrate (upper graphs) and silver nanoparticles (bottom graphs) exposures. Ag_{tot}- total silver; Ag_{dis}- dissolved silver; Ag_{ionic}- ionic silver; for more details on the fractions please see the Material and Methods section.

Chapter 4

Bioaccumulation of silver nanoparticles by *Daphnia magna*

4 Silver nanoparticles are highly (bio)accumulated by *Daphnia magna* under environmental relevant conditions

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Abstract

Silver nanoparticles (AgNP) are used by industry and medicine as an antimicrobial agent. The production and incorporation of AgNP into products can potentially lead to their entrance in the aquatic environment. It is of currently knowledge that the toxicity of AgNP is mostly associated to the dissolution of Ag ions (Ag^+) from nanoparticles and that Ag^+ are more toxic than AgNP to aquatic species. The toxicity assessment however takes into consideration single routes of exposure, and is done under non-realistic environmental concentrations; thus, underestimating the actual threat that AgNP may induce in the environment. In this study we chose to use bioconcentration as endpoint to compare the potential of *Daphnia magna* to accumulate either AgNP or $AgNO_3$ from different exposures routes: *a)* water, *b)* food and *c)* water and food. Concentrations of exposure to both AgNP and $AgNO_3$ were calculated based on acute tests, and were chose as the concentration not causing a lethal effect on Daphnia. The one-compartment kinetic model was applied to obtain uptake (k1), elimination (k2) constant rates and bioconcentration or bioaccumulation factors (BCF or BAF).

Our results demonstrate that $AgNO_3$ induced a higher BCF in Daphnia when water was the only source of exposure. There was no difference in the Ag bioaccumulation pattern when comparing both AgNP and $AgNO_3$ exposures via food, and finally, by reproducing the worse-case scenario in a contaminated water and food exposures, AgNPs induced a higher bioaccumulation factor (BAF) in Daphnia.

4.1 Introduction

A nanomaterial is recommended by the European Union to be defined as "a natural, incidental or manufactured material containing particles in an unbounded state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range of 1nm-100nm" (EU, 2011a).

The nanomaterial size functionalization, i.e., the variety of novel applications related to their specific small size-range is the major responsible for the great application of nanoparticles in industry (Fabrega, Luoma, Tyler, Galloway, & Lead, 2011). Silver nanoparticles (AgNP) is listed as the most applied metal-based nanoparticle nowadays into consumer products. Because of its inherent properties such as catalytic efficiency, thermal conductivity (Capek, 2004) and antimicrobial activity (Morones, Elechiguerra, & Camacho, 2005; Sondi & Salopek-Sondi, 2004), AgNP may be found into daily care products, clothing, sport gears, food packaging and hospital supplies (Benn & Westerhoff, 2008; Li, Wu, & Ong, 2005). The manufacturing process and wide usage of silver nanoparticles lead to the entrance of AgNP in the various environmental compartments as air, soil and waters (Blaser, Scheringer, MacLeod, & Hungerbühler, 2008). For the purpose of this study we will focus on the aquatic compartment. According to Yu et al (2012), there are several routes of entry of AgNP into the aquatic environment: *a)* through washing of AgNP-containing products, which will leach nanoparticles into the environment; *b)* air suspended AgNP that will finally deposit in water and *c)* runoff of AgNP from polluted soils into adjacent surface waters. Either way Ag will be present in the aquatic

environment, as nanoparticles or as their transformation products, and since silver is a non-essential metal for aquatic biota, there is an urgent need to investigate the inherent toxicity effects to aquatic species.

Nonetheless, it is important to bear in mind that the composition of natural waters will change the original characteristics of AgNP. Once present in the environment AgNP will likely to undergo agglomeration due to the surface forces acting among particles (Elzey & Grassian, 2009), interact with chlorides, sulphates and natural organic matter (NOM) (Lee, Fortin, & Campbell, 2004) and/or undergo dissolution through oxidative process, in which Ag^{+} ions will be released from nanoparticles (Ho, Yau, Lok, So, & Che, 2010). Those interactions are highly depended on the surface functionalization of the nanoparticles as well as the surrounding media composition. Therefore, the toxicity assessment of AgNP, conducted under laboratory conditions, may overestimate the actual risk posed by AgNP to aquatic species. Those effects are evaluated in key species, occupying different levels of the trophic chain, such as the green algae *P. subcapitata, C. reinhardti,* and *C. vulgaris* (Hiriart-Baer, Fortin, Lee, & Campbell, 2006; Navarro et al., 2008), zooplankton species (Daphnia sp.) (Heinlaan et al., 2011; Ribeiro et al., 2014; Zhao & Wang, 2011) and fish (e.g. *Danio rerio* and *Oncorhynchus mykiss),* among other species (Asharani, Lian Wu, Gong, & Valiyaveettil, 2008; Scown et al., 2010; Wood, Hogstrand, Galvez, & Munger, 1996a). Unfortunately, the ecotoxicity assessment of AgNP to those species is conducted under non-realistic exposure scenarios, not only due to the controlled laboratory conditions but also due to the use of unlikely occurring concentrations.

In this study we focused on *Daphnia magna* as our model species to study the

bioconcentration and bioaccumulation of AgNP in comparison with $AgNO_3$, which was used as an ionic control, with the interest of better estimate the potential for bioconcentration of AgNP in comparison to its ionic form. Bioconcentration and bioaccumulation were chosen as endpoints here because they provide a more conservative response rather than lethal effect concentrations (LC_{50}) and might enable the prediction of risk under chemical doses that are sometimes taken as conservative concentrations. We previously demonstrated that Ag induces various toxic effects on *D. magna* such as mortality, reduced reproduction and decreased feeding rates (Ribeiro et al., 2014) however those results were observed based on total silver concentrations without actually knowing how the bioavailable fraction of Ag interfered with these responses.

The bioaccumulation approach though will focus on the chemical fraction that is actually available for the animals, thus the bioavailability. The term bioconcentration will hereafter be used to describe uptake and elimination of silver by *D. magna* through passive diffusion from the surrounding media (i.e., water) whereas bioaccumulation will include other routes of exposure such as food or when both water and food exposures are present.

Because silver is a very reactive metal, its bioavailability in solution is crucial to understand the interactions with organisms and consequently biological effects it may induce. According to the Biotic Ligand Model (BLM), uptake and toxicity of silver should fluctuate as a function of free Ag^+ in solution (Paquin, Santore, Wu, & Kavvadas, 2000). However, it is unknown how the different sources of Ag (nanoparticle and ionic) will induce bioaccumulation. The BLM predicts toxicity induced by the free Ag ion, but bioaccumulation in Daphnia may be also induced

by the intake of Ag complexes in solution and AgNP agglomerates/aggregates, as well as feeding on Ag-internalized algae. In this study we intended to reproduce the most probable routes of AgNP internalization by *Daphnia magna*, and compare between bioaccumulation factors (BAF) which of these routes may indicate a worse case scenario. In addition, the same approach was reproduced for $AgNO_3$ in order to compare BAF´s between nanoparticle and ionic silver exposures. Figure 4.8 gives an overview of the different routes of uptake of silver nanoparticles and ionic silver by *Daphnia magna* as well as their interaction in the media.

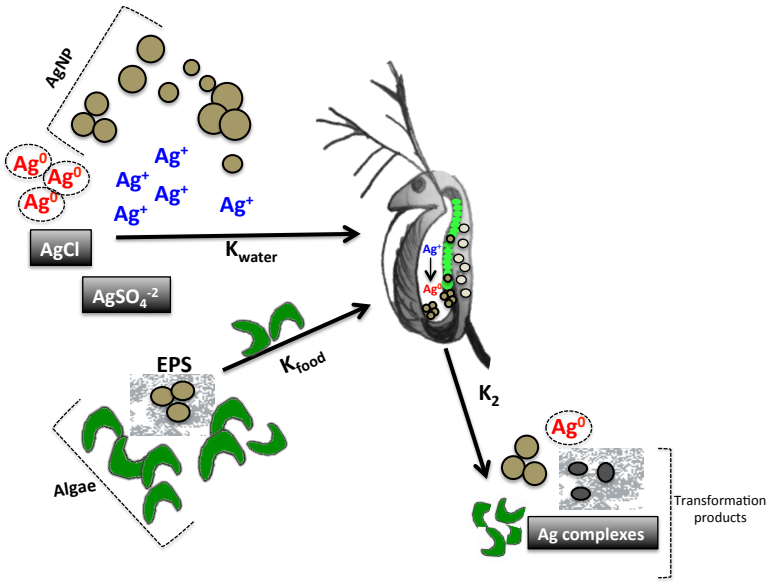

Figure 4.8. Schematic representation of the various routes of uptake of AgNP and Ag^+ by *Daphnia magna*. AgNP is present in the surrounding environment as single particles and particle agglomerates and/or aggregates. Ionic silver (Ag^+) is originated from AgNP dissolution and it is highly reactive with other components of the media such as Cl^- and SO_4^{-2}, forming dissolved silver complexes. Daphnia is susceptible on algae feeding that have previously internalized dissolved Ag. The presence of algae creates an exopolymeric substance (EPS) that induces AgNP agglomeration, which in turn will be also ingested by Daphnia. Inside the organism, the following reactions are likely to occur: AgNP may dissolve, due to the acid environment of the gut, thus releasing ionic Ag^+; Daphnia may transform ionic Ag^+ into metallic Ag^0; AgNP agglomerates may not be completely excreted, depending on their size. Those reactions will likely induce AgNP to be concentrated into the Daphnia body, with possible trophic transfer. k_{water} is the constant of uptake from water, k_{food} is the constant uptake from food and k_2 is the constant of elimination.

4.2 Methods

Daphnia magna culture

Daphnia magna (Clone K6 - Antwerp, Belgium) has been kept under controlled conditions in the laboratory for over 6 years. Adult Daphnia was cultured in ASTM hard water (ASTM, 1980) with seaweed extract and the green algae *Pseudokirchneriella subpcapitata* provided as food. Adults were maintained in 1L jars (30 individuals/jar) and the medium renewed 3 times a week. The broods used for ecotoxicological testing were from the 3rd to 5th brood, and subsequent broods were used to renew the culture.

Chemicals

Silver nitrate ($AgNO_3$), used as an ionic control in this study, was purchased from Sigma-Aldrich as a crystalline powder, 99% purity CAS 7761-88-8.

Nanoparticle characterization

Silver nanoparticles (AgNP) used in this study were manufactured by AMEPOX (Poland). AgNP initial batch was provided as a colloidal suspension in water (1000mg/L), without surfactant and with an initial particle size of 3-8nm, coated with an alkane material.

The Z-average hydrodynamic diameter of AgNP was measured over two weeks in ultra-pure water at a concentration of 10 mg/L, using a Malvern Zetasizer. Up to 18 replicates were taken to gain a robust value for the mean-Z-average hydrodynamic diameter. The Z-average diameter of NP was also measured in ASTM suspension during long (4 days) and short (12 minutes) periods to study

the agglomeration behavior of AgNP in our test-media. The initial stock nanoparticles suspension was diluted with the ASTM media to the concentration of 1 mg/L in normal DLS cuvettes and inserted immediately in the instrument. The measurement was started at a fixed attenuator and measurement position to avoid the optimization time, the correlation time was set to 2 s and 120 data points were generally obtained. For the long-term experiments (days) the first measurement (day zero) was obtained by creating an average result from the short-term data points. The cuvettes were stored in the dark and three measurements were performed (3 runs of 20 s each) in the following days. To evaluate the effect of particles sedimentation, samples were shaken after performing the measurement and a new measurement was done.

Derived count rates were included in the long-term experiments to compare the capacity of the remaining particles (large and small) to scatter light.

For TEM imaging, the initial suspension of AgNP (1000 mg/L) was diluted to 0.1 mg/mL in ASTM, and a drop of this suspension was deposited on a holey carbon coated Cu TEM grid and dried at room temperature for several hours before examination. Experiments were carried out on a JEOL 2010 analytical TEM with a resolution of 0.19 nm, an electron probe size down to 0.5 nm and a maximum specimen tilt of ±10° along both axes. The instrument was equipped with an Oxford Instruments LZ5 windowless energy dispersive X-ray spectrometer (EDS) controlled by INCA.

Bioconcentration tests

Eight to ten days-old *Daphnia magna* were exposed to silver nanoparticles and silver nitrate in ASTM media for 48 hours (uptake phase) and another 48 hours under clean media, for the elimination phase. Concentrations of exposure were chosen based on EC (Effect Concentration) values from previous experiments (Ribeiro et al., 2014). For AgNP, Daphnia were exposed to 5µg/L and 10µg/L, which corresponded NOEC (No-Observed-Effect-Concentration) from acute test. For $AgNO_3$, those corresponding concentrations were 0.25µg/L and 0.5µg/L. The test was conducted as follows: 3 replicates for each Ag form and concentration were performed, and sampling of animals and water was carried out at time 0 (control), 3h, 6h, 12h, 24h, 36h, 48h, 72h and 96h. At each sampling point, 15 daphnia were collected from each replicate jar and washed with Milli-Q water using a small sieve. This step aimed at washing out any loosely adsorbed NP from the carapace. Daphnia was then transferred to eppendorff tubes and dried in the oven at 50ºC overnight.

Bioaccumulation tests

For dietary exposures, *P. subcapitata* in later exponential growth phase were spiked with AgNP and $AgNO_3$ for 48h at a concentration of 15 mg/L, equivalent to the EC_{10}, based on the growth inhibition assay of *R. subcapitata* (Ribeiro et al., 2014) and on bioconcentration factors previously obtained for algae (Chapter 3). Following exposure to both AgNP and $AgNO_3$, algae was then separated from the solution by centrifugation and re-suspended in ASTM media to provide a cell concentration of $3x10^5$ algae cells/ml/Daphnia. The internal Ag concentration in algae after the exposure to AgNP was 27.71 mg/g (dw) and to $AgNO_3$ it was 23.67

mg/g (dw). For the purpose of comparison between exposures via food and exposures via water and water + food, the internal concentration in algae, in mg/g (dw) was transformed into mg/L, by considering the volume of exposure and the wet weight of algae. After transformation, the equivalent concentration of Ag in algae media was then 21.61 mg/L (in the AgNP exposure media) and 18.46 mg/L (in the $AgNO_3$ exposure media) at the beginning of exposure. Water was sampled together with the animal sampling at different time points, to measure Ag concentration in water, originated from algae. Sampling time-points were the same as described above for the bioconcentration tests.

A third experimental design was performed in which Daphnia were exposed to both contaminated water and food. For these tests, the concentration of AgNP and $AgNO_3$ in the water were the lower concentration applied in the bioconcentration testes, i.e., 5µg/L for AgNP and 0.25µg/L for $AgNO_3$. Algae were dosed with the same concentration from the dietary exposure (15 µg/L of $AgNO_3$ and AgNP). The experimental design was similar for all routes of exposure and it was constituted by 3 replicates of 2L jars with ASTM for each AgNP and $AgNO_3$ treatment, with 120 animals per jar. Test was conducted under controlled conditions of temperature (20ºC±1ºC) and photoperiod (16h-8h light-dark). Sampling time points for animal and water were the same as described for bioconcentration test. The remaining algae that was used to feed Daphnia on both experimental conditions was collected in triplicate on a 50mL falcon tube and centrifuged to separate algae from the media. After centrifugation, the supernatant was discharged and the algae pellet was dried at 50ºC to a constant weight then weighted and digested for its total Ag content measurement.

Sample digestion

Water samples were digested according to the method described by Ribeiro et al. (2014). Briefly, samples were evaporated inside a Teflon beaker, on a hotplate without boiling, until 1ml of sample was remained. Aqua regia was then added and samples were heated again until 1ml was left.

In order to measure the concentration of Ag inside *D. magna*, and *P. subcapitata*, sample dry tissues were weighted and transferred to Teflon beakers for digestion. 3ml of HNO_3 (65% trace analysis) was added to the samples and heated on a hotplate for about 30min with their lids on. After this, 1ml of HCl was mixed in the beakers and those were replaced on the hotplate for a further period of 30min. Subsequently, the lids were removed and the acid was allowed to evaporate (without boiling) until 1-1.5ml remained in the beakers. Samples were then cooled at room temperature and dilute with 1% HCl to a final volume of 45ml for further analysis of Ag content. Ag measurements were conducted by Atomic Absorption Spectroscopy (AAS).

Toxicokinetics modeling

A one-compartment model was used to describe the toxicokinetics of AgNP and $AgNO_3$ in *Daphnia magna*. This model describes the kinetics of Ag based on mass balance equations. Assuming that concentration of exposure is constant, two types of equations were used to model uptake and elimination of AgNP and $AgNO_3$ in Daphnia, as described below:

$$Q(t) = \frac{k1}{k2} \times Cexp. \times (1 - e^{(-k2*t)}) \quad (1) - \textit{Uptake equation}$$

$$Q(t) = \frac{\mathrm{k1}}{\mathrm{k2}} \times \mathrm{C}exp. \times (1 - e^{(-\mathrm{k2} * (\mathrm{t} - \mathrm{tc}))} - e(^{-k2 \times t)}) \quad (2) - \textit{Elimination equation}$$

Where:

Q (t) is the concentration in the organism at time *t* (µg/g dry weight)

k_1 is the uptake constant (L/g/hour)

k_2 is the elimination constant (1/hour)

Cexp is the concentration of exposure (µg/L)

In the case of exposure via water, a bioconcentration factor (BCF) was calculated as the ratio between k_1 and k_2 and also as the ration between the average maximum internal concentration in Daphnia and concentration in water.

The model described above was used to interpret kinetics of AgNP and $AgNO_3$ in *D. magna* through water exposure. However, in order to assess the contribution of food as a Ag source and the join exposure routes by contaminated water and food, additional parameters should be considered in the model. Therefore, dietary exposure was modeled according to the follow:

$$Q(t) = \frac{k1f.cf}{k2} \times (1 - e^{-k2 \times t)}$$

Where:

k_1f – Constant of uptake from food ($g_{algae}/g_{daphnia}$/hour)

Cf - Concentration in food (µg Ag/g algae dry weight)

The contribution of water and food to the kinetics of Ag was modeled as:

$$Q(t) = \frac{kw \times cw + kf \times cf}{k2} \times (1 - e^{-k2 \times t)}$$

Where:

kw – constant of uptake from water (L/g/hour)

cw – Concentration in the water (µg/L)

kf – constant of uptake from food ($g_{algae}/g_{daphnia}$/hour)

cf – Concentration in the food (µg Ag/g algae dry weight)

In both cases, a bioaccumulation factor (BAF) was calculated by the ratio between the concentration in the organism and the total concentration of Ag in water. For that, the total Ag in food was transformed in a concentration of Ag in water, by using the total amount of Ag in algae divided by the volume of (media) exposure; therefore, when Ag was present in food (algae) and water, a total concentration expressed as mg Ag/L was possible to calculate, by summing the concentration in water and the concentration in food, previously transformed as mg Ag per volume of water.

4.3 Results

Particle characterization

The hydrodynamic diameter of the AgNP in water was reasonably stable for the duration of the experiment with a size ranging from 127 to 132 nm (Fig. 4.1*a* and *b*). Moreover, Z-average size of AgNP at a concentration of 1 mg/L was followed over a short and long-term period in ASTM media. Although this concentration is not relevant for the toxicokinetics experiments, it was used as

the lowest concentration providing statistically significant results from the DLS analysis. Figures 4.1*b* and 4.1*d* shows that in ASTM, the zeta-average diameters of particles agglomerates at day zero was approximately 80 nm and after one day of experiment the zeta average increased to ~200 nm, denoting that large agglomerates are starting to impact the scattered intensity weighted diameter (zeta-average). Agglomerates were larger after 3 days, reaching 350 nm.

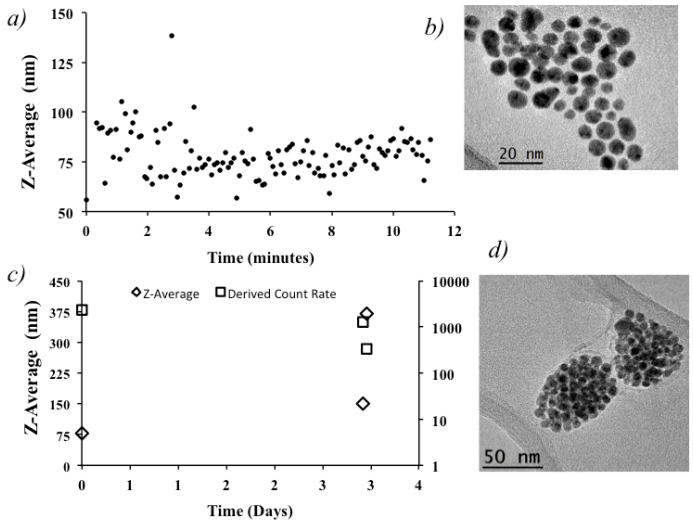

Figure 4.1. Z-average hydrodynamic diameter of silver nanoparticles (AgNP) in *a)* short-term experiments and *c)* long-term experiment. Pictures *b* and *d* shows TEM images of AgNP in water and in ASTM media, respectively.

Toxicokinetics

Route of exposure: Water

Ag exposure measurements in water were carried out along with daphnia sampling, with the objective of monitoring Ag concentrations in time. For $AgNO_3$ exposures, the samples from the lower exposure concentration (0.25mg/L) were

below the instrument detection limit (0.138 mg/L). For exposure concentration of 0.5 mg/L the measurements of Ag were within 90% recovery from the nominal concentration, along time (data not presented).

Regarding measured concentrations in water from AgNP exposure, those are presented in figure 4.2. For AgNP we were able to measure total and dissolved concentrations, i.e., ionic Ag released from AgNP. However, as shown in figure 4.2, there were a few gaps in the measured concentration with time, meaning that Ag (either total and ionic) was sometimes present in the media at concentrations below the detection limit. The total measured Ag at 10 µg/L of AgNP remained roughly constant during the uptake phase whereas the concentration of ionic Ag released from AgNP increased from time zero to 48 hours of exposure at both concentrations of 5 µg/L and 10 µg/L.

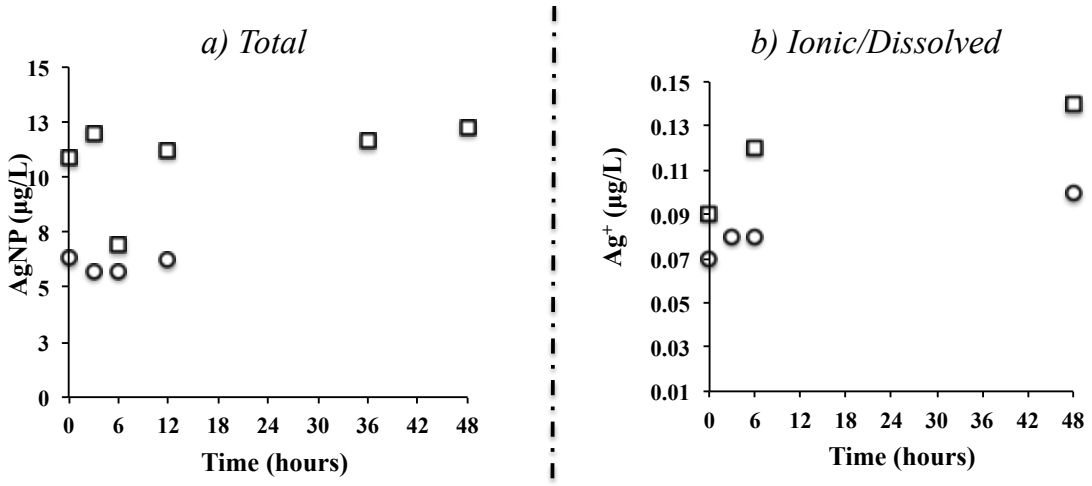

Figure 4.2. Concentrations of a) total and b) dissolved Ag (mg/L) in AgNP water exposure media (○- 5 mg/L and □- 10 mg/L) measured for a 48h period.

$AgNO_3$ induced a higher bioconcentration factor in *D. magna* when compared to AgNP, based on both calculation methods. However, at AgNP treatments the differences between BCF(1) and BCF(2) were smaller than at $AgNO_3$. In addition, when Daphnia was exposed to AgNP, they achieved an average maximum internal concentration at 48h of exposure at both 5 μg/L and 10 μg/L, which were 30.90μg/g (dw) and 34.81μg/g (dw), respectively (table 4.3). Still, when exposure was carried out with $AgNO_3$, the time needed to reach a maximum internal concentration by Daphnia was different for different exposure concentrations: at 0.25 μg/L of $AgNO_3$, Daphnia achieved a maximum internal concentration of 17.68 μg/g (dw) at 6h of exposure, while under 0.5 μg/L, this concentration was 24.95 μg/g (dw) and which was reached after 24h of exposure (table 4.3).

Regarding the parameters values estimated by the kinetics model, present in table 4.1, higher values for constant of uptake (k1) were observed under $AgNO_3$ exposure concentration, whereas elimination rate constant varied according to the Ag form (nanoparticle and ionic) and concentration (table 4.1).

Interestingly, at 0.5μg/L $AgNO_3$, the uptake (k_1) and elimination (k_2) constants were 10-fold lower than at 0.25μg/L however the BCF value was statistically equivalent (table 4.1).

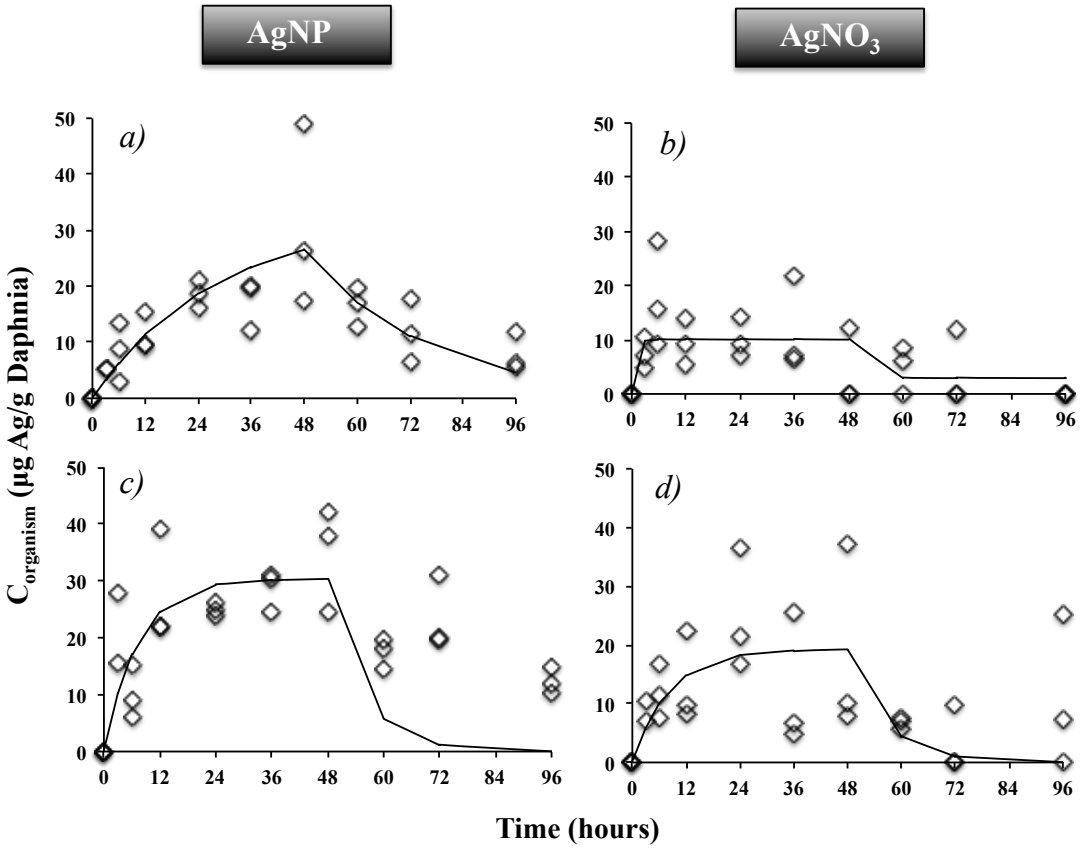

Figure 4.3. Uptake and elimination kinetics of silver in *Daphnia magna* exposed through water to *a)* 5 µg/L and *c)* 10 µg/L of AgNP, and to *b)* 0.25 µg/L and *d)* 0.5 µg/L of $AgNO_3$. The kinetic model curve was derived by equation 1 for the uptake phase and equation 2 for the elimination phase, using Daphnia Ag body burdens. The elimination phase started at 48h.

Table 4.1. Kinetics parameters estimated for *Daphnia magna* exposures to AgNP and $AgNO_3$ through water. k_1 is the constant of uptake, k_2 is the constant of elimination, BCF (1) is the bioconcentration factor, calculated as the ratio between k1 and k2 and BCF (2) is the bioconcentration factor calculated as the ratio between the average maximum concentration in the organism and concentration in water.

Model parameters	AgNP 5µg/L	AgNP 10µg/L	$AgNO_3$ 0.25µg/L	$AgNO_3$ 0.5µg/L
k1	0.23	0.42	50.4	4.74
k2	0.04	0.13	1.3	0.12
BCF (1)	5.75	3.23	38.76	39.05
BCF (2)	6.18	3.48	70.72	49.90

Route of exposure: food

Figure 4.4 shows the variation of Ag concentrations in the media with time, when food was provided as the only source of Ag to Daphnia. This concentration was calculated considering the number of algae cells per milliliter of solution, and the wet weight of algae. The Ag measured concentrations in water in this experimental approach was assumed to be originated from algae. For both AgNP and $AgNO_3$, concentrations of total Ag in water decreased until 6h of exposure and after this period, Ag in the media fluctuates with time, however those fluctuations in concentration were greater on the AgNP media.

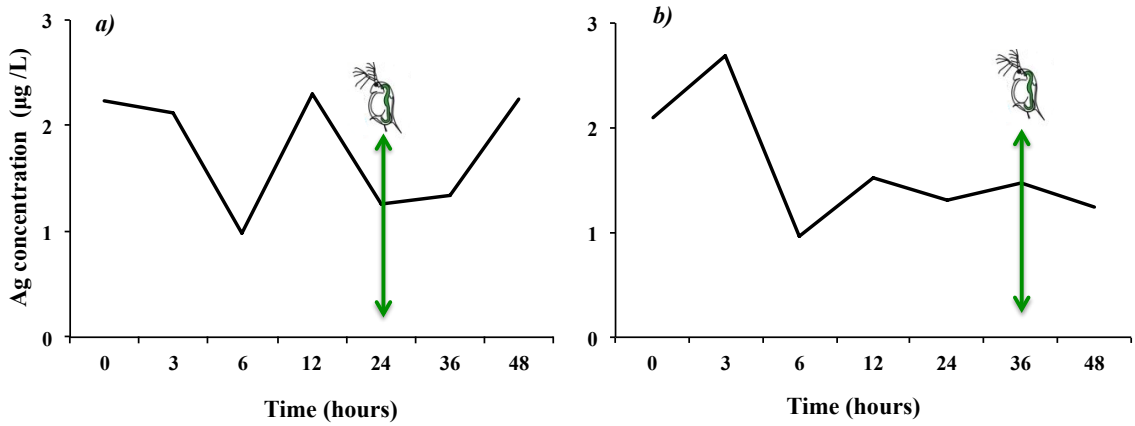

Figure 4.4. Concentration of Ag in contaminated algae, expressed as µg/L, in time: *a*) AgNP exposure and *b*) $AgNO_3$ exposure. The arrows indicate the time at which *Daphnia magna* achieved maximum Ag body burden.

Regarding body concentrations of Ag in *D. magna*, no difference was detected in the amount of internal Ag concentration regarding exposures to AgNP and $AgNO_3$. On both treatments, animals reached a similar value of body burden. Anyhow, Daphnia fed on algae containing AgNP reached an internal maximum concentration of 20.51 µg/g (dw) (SE=4.57) at 24h of exposure; while Daphnia fed on $AgNO_3$ contaminated algae reached its maximum internal concentration of 20.13 µg/g (dw) (SE=2.96) at 36h of exposure (fig. 4.5, table 4.3).

However, the constant of uptake (k1) was slightly higher at AgNP treatment as well as the BAF value (table 4.2).

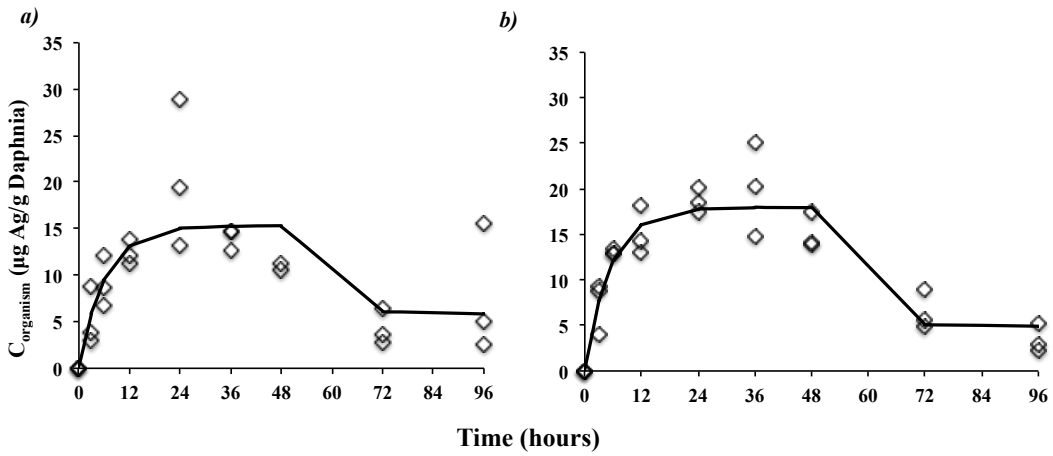

Figure 4.5. Uptake and elimination kinetics of silver in *Daphnia magna* exposed via food to *a)* AgNP and *b)* $AgNO_3$. The kinetic model curve was calculated by equation 1 for the uptake phase and equation 2 for the elimination phase, using Daphnia body burdens. The elimination phase started at 48h.

Route of exposure: water and food

The total Ag concentrations for this exposure route were measured in time, considering concentration in algae plus concentration in water as the total Ag source for Daphnia (expressed as mg/L). Figure 4.6 demonstrate that the total Ag concentration in the AgNP exposure was 10-fold higher compared to $AgNO_3$ exposure media, and showed a different trend on Ag decay behavior between both treatments. While AgNP had a gradual decrease on Ag concentration with time, reaching its lower concentration at 48h of exposure, $AgNO_3$ showed an abrupt decrease during the first 12h and afterwards Ag concentration increased again and varied with time.

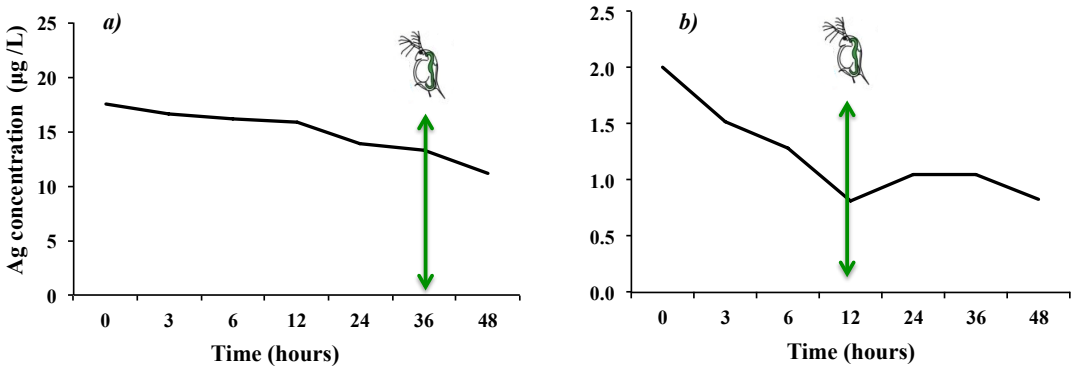

Figure 4.6. Total Ag concentration from contaminated water and algae, expressed as µg/L, in time: *a)* AgNP exposure and *b)* $AgNO_3$ exposure. The arrows indicate the time that *Daphnia magna* needed to achieve maximum Ag body burden. Note the 10-fold difference on the x-axis between figures *a* and *b*.

By exposing Daphnia to water and food as sources of contamination, animals dosed with AgNP were able to uptake up to twice the amount of Ag than animals treated with $AgNO_3$ (fig.4.7).

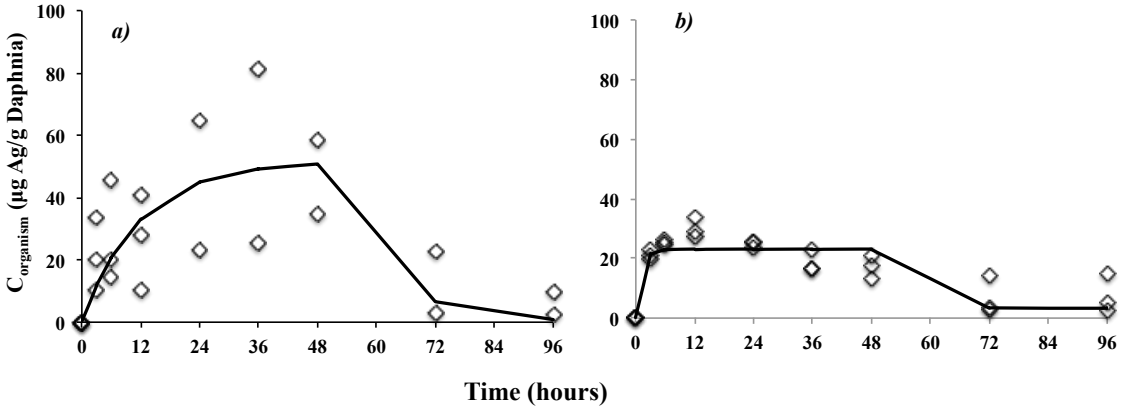

Figure 4.7. Uptake and elimination kinetics of silver in *Daphnia magna* exposed through water and food to *a)* AgNP and *b)* $AgNO_3$. The kinetic model curve was calculated by equation 1 for the uptake phase and equation 2 for the elimination phase, using Daphnia body burdens. The elimination phase started at 48h.

Likewise, the Bioaccumulation factor (BAF), calculated as the maximum Ag concentration in Daphnia divided by the total Ag exposure concentration (from water and food, in µg/L) was at AgNP exposure than at $AgNO_3$ (table 4.2). The Ag

maximum body burden concentration on the AgNP exposure was 53.25 µg/g (dw) (SE=17.24), achieved at 36h of exposure while on the $AgNO_3$, Daphnia reached a maximum Ag body burden of 30.06 µg/g (dw) (SE=1.90) at 12h of exposure (table 4.3). The model parameters for this exposure were all higher in the AgNP treatment, except for the uptake rate (k_1), which was higher on $AgNO_3$ exposure (table 4.2).

Table 4.2. Kinetics parameters estimate for the exposure of *D. magna* to AgNP and $AgNO_3$ through food, and water + food. k_1 is the constant of uptake, k_2 is the constant of elimination, A is the assimilation efficiency and BAF is the bioaccumulation factor, calculated as the ratio between the concentration in the organism and the concentration in water.

Exposure route	Parameters	AgNP	$AgNO_3$
Food	K_1	0.09	0.14
	K_2	0.16	0.19
	A	2.49	3.38
	BAF	**0.74**	**0.85**
Water & Food	K_{water}	0.73	0.20
	K_{food}	0.016	0.38
	k_2	0.09	0.87
	BAF	**1.40**	**0.73**

4.4 Discussion

In this study we aimed at comparing the Ag bioconcentration and bioaccumulation pattern in *Daphnia magna* when exposed through different routes of contamination (water and/or food), and to different Ag forms (AgNP and $AgNO_3$). Differences in BCF and BAF were noted for different routes of exposure and concentrations that were applied in this study.

Uptake and elimination constant rates calculated for AgNP exposures through water were extremely lower when compared to those of $AgNO_3$, generating a lower BCF. When considering exposure concentrations, this was not expected, as Ag concentrations from AgNP were 20 times higher than those from $AgNO_3$ (based on Ag).

The model parameters for the AgNP exposure indicated that the constant of uptake and elimination where inversely proportional to the concentration of AgNP in solution: at 5µg/L of AgNP, uptake rate constant (k1) was two-fold lower and the elimination rate constant (k2) was three-fold lower than at an exposure of 10µg/L.

Those values may be a reflection of the chemical behavior of nanoparticles in solution, and their interaction with Daphnia. Particle agglomeration tends to be higher at higher particle concentration due to the increased collision between them (Jiang, Oberdörster, & Biswas, 2008). In *Daphnia magna*, the ingestion and concentration of particles and compounds in the body is related to their feeding activity. Since they can filter a large amount of water compared to their body size, it is likely that particles ranging in size from 0.4 to 40µm will be ingested (Gophen & Geller, 1984). This means that nanoparticle agglomerates varying in

size are likely to be ingested by Daphnia. Assuming that at a concentration of 10µg/L of AgNP, the hydrodynamic diameter of particle agglomerates was bigger than at 5 µg/L, once ingested, those agglomerates were faster excreted by Daphnia, therefore a higher elimination rate constant was predicted at this concentration. *Daphnia magna* exposed to titanium dioxide nanoparticles (TiO_2-NP), which were assumed to be in the size range of 50µm agglomerates also showed a lower elimination rate, and a considerable amount of TiO_2-NP was still observed in their gut after a 72h period of depuration (Zhu, Chang, & Chen, 2010). It is believed that bigger particles will enter on the digestive system, in which food particles are taken into the peritrophic membrane and lately absorbed by the gut epithelium (Gophen & Geller, 1984). Unlike $AgNO_3$, different concentrations of AgNP induced differences in the BCF value on *D. magna*. Those were higher at the low concentration of AgNP, mainly due to the low elimination rate. Moreover, in the NP exposure, Daphnia reached a maximum body burden concentration closer to the concentration that was expected under a steady state. Regarding $AgNO_3$ exposure, Daphnia dosed with 0.25µg/L of Ag (as $AgNO_3$) had the highest value of uptake constant, compared to all exposure scenarios through water, or even compared to 0.5µg/L of Ag (as $AgNO_3$), as presented in table 1.

Considering the kinetics of Ag in the $AgNO_3$ exposure media, Ag levels remained within the nominal range of 0.5 µg/L during the 48h exposure period. Samples from the 0.25 µg/L Ag were not detected by the AAS instrument.

Table 4.3. Time needed to achieve maximum Ag body burden in *Daphnia magna*, according to the route of exposure of AgNP and $AgNO_3$

	Route of exposure	Time (h)	body burden (max)**
AgNP	**Water***	48	30.9 (9.38)
	Food	24	20.51 (4.57)
	Water + Food	36	53.25 (17.24)
$AgNO_3$	**Water**	6	17.68 (5.57)
	Food	36	20.13 (2.96)
	Water + Food	12	30.06 (1.90)

*Concentration in water: 0.25µg/L **µg/g/dw

The kinetics parameters estimated by the uptake and elimination equations (presented in table 4.1) indicate that at 0.25 µg Ag /L of, the uptake rate constant k1 is much higher when compared to the k1 estimated for the 0.25 µg Ag /L exposure, as well as the elimination rate constant. Moreover, the difference between the BCF (1) and BCF (2) is greater at 0.25 µg Ag/L exposure, which suggests that under this exposure concentration, Daphnia was far from achieving a steady state phase. This may be related to the speciation of Ag in the media, which is dependent on the Ag concentration. The lower the concentration of Ag in solution, the lower will be the chances for speciation and chemical complexation with other compounds in the media (such as chlorides or sulphates) and thus higher will be the bioavailability of Ag to Daphnia (Lam & Wang, 2006). Since Ag concentrations at the 0.25 µg/L exposure level were below the analytical detection limit, it was expected that all the Ag present in the

media had been ingested by Daphnia, at a higher rate of uptake, which also explains the fact that at this concentration Daphnia reached the maximum Ag body burden after 6 hours of exposure, and that concentration was far from being the steady state concentration. On the other hand, at a double dose of Ag in the exposure media (0.5 µg/L), Daphnids reached their maximum Ag body burden after 24 hours of exposure, and that value was closer from the value expected for body burden at steady state. Nonetheless, the BCF values calculated for both exposure concentrations of Ag as $AgNO_3$ were similar, which indicates that the Ag concentration in solution does not influence the BCF but it does plays a role on the kinetics of uptake and elimination.

The assessment of Ag bioaccumulation through food revealed that regardless the form in which silver was present in the food (as AgNP or $AgNO_3$), the Bioaccumulation Factor and the Ag kinetics in Daphnia were similar, as shown in figure 4.5. The difference between exposures was that by feeding on AgNP-contaminated algae, Daphnia reached its maximum Ag body burden at 24h of exposure, whereas under $AgNO_3$ exposures, the maximum Ag body burden was achieved after 36h of exposure. However, these maximum values of Ag body concentration in Daphnia were very similar between exposures: 20.51µg/g (dry weight) for AgNP and 20.13µg/g (dry weight) for $AgNO_3$.

To our knowledge, till now there is no evidence that freshwater algae species such as *Raphidocelis subcapitata* internalize AgNPs as single particles or agglomerates (Franklin et al., 2007; Ma, Geiser-Lee, Deng, & Kolmakov, 2010). In addition, our observations shown in Chapter 3 highlight that Ag detected in algae was mainly originated through the ionic uptake, i.e., uptake of free Ag^+ in solution by algae. Moreover, Ag concentrations measured in algae were 27.71

µg/g (dry weight) in algae previously treated with AgNP and 23.67 µg/g (dry weight) in algae dosed with $AgNO_3$. Given that, it was already expected that the concentration of Ag exposure through diet would be similar between AgNP and $AgNO_3$ as well as the kinetics curves for this route of exposure.

On the other hand, the kinetics of Ag concentration in solution in which Ag was present both in water and in the algae cells was different from the media in which Ag was present as contaminated algae only. In figure 4.6 it can be seen that while Ag concentration in AgNP exposure media remained more or less constant throughout exposure, on $AgNO_3$ there was an abrupt decrease in concentration during the first 12h of exposure followed by the increase in the Ag levels. Regarding the kinetic parameters of Daphnia, we observed that at the AgNP exposure, the constant of uptake from water (K_{water}) was much higher than the constant of uptake from food (K_{food}). This suggests that the ingestion of AgNP in Daphnia was driven by the nanoparticles suspension in solution. Indeed, because it is suggested that *R. subcapitata* cannot internalize nanoparticles (Franklin et al., 2007), (Chapter 3) it was expected that the contribution of diet in the uptake of AgNP by Daphnia would be low. Moreover, the presence of nanoparticles in solution is known to negatively influence the feeding behavior of Daphnia (Lopes et al., 2013; Ribeiro et al., 2014; Zhu et al., 2010), which can be associated with the low k_{food} predicted for this exposure. The presence of algae in the media also played a role on the accumulation of AgNP by Daphnia. Algae segregates exopolymeric substances (EPS) that can make particles either agglomerate, stabilize or dissolve in the media (Miao et al., 2009; Zhang et al., 2013). Considering that nanoparticle agglomeration is a dynamic process, and Daphnia filter feeding behavior is a constant mechanism, we hypothesized that

nanoparticles agglomerates were surrounding the algae cells due to the EPS influence and consequently those agglomerates were ingested with algae by the appendage filtering of Daphnia into their gut. The model prediction of a low value for k_2 suggests that the excretion of the ingested agglomerates was somehow unsuccessful, causing particles to be accumulated into the body of Daphnia (Geller & Müller, 1981; Zhu et al., 2010).

The exposure of Daphnia to $AgNO_3$ via water and food though revealed a different scenario: while on AgNP Daphnia had a k_{water} much higher than the k_{food}, under $AgNO_3$ exposure we observed the opposite. This indicates that the diet played a major role on the uptake of Ag by Daphnia. By feeding on algae cells that have internalized ionic Ag (Ag^+) and/or dissolved Ag complexes (Lee, Fortin, & Campbell, 2005) the chances of Ag body burden were increased. Although the kinetic model predicted a higher K_{food} compared to k_{water} on the $AgNO_3$ media, the difference between those two parameters was not as big as it was for the AgNP media. Therefore, uptake from water was also significant to induce bioaccumulation of Ag by Daphnia, which was probably due to the presence of ionic Ag^+ and Ag dissolved species (AgCl, $AgSO_4^{-2}$) in solution, which were expected to be higher than in AgNP exposure media. The mechanism of ionic Ag^+ uptake in Daphnia involves passive diffusion of ions from the surrounding media, by Ag^+ affinity with the biological channels that perform osmoregulation in Daphnids (Bianchini & Wood, 2003; Wood, Hogstrand, Galvez, & Munger, 1996b), whereas the uptake of larger particle involves different mechanisms including dissociation, permeation and active transports (Zhao & Wang, 2010). Because of the readiness in which ionic Ag^+ and dissolved Ag species are taken up by *Daphnia* under $AgNO_3$ exposure and because algae with internalized Ag

had a greater contribution in the Ag body burden of Daphnia, it was expected that the elimination of these variable Ag forms would be more efficient then it was in the nanoparticle exposure. Therefore, under $AgNO_3$ exposure, Ag is probable eliminated in the feces and through the peristaltic movement of the gut, which may not happen by the same mechanisms for the elimination of AgNP clusters. It is therefore expected that the combination of these factors lead to a higher Bioaccumulation Factor (BAF) in *Daphnia magna* when they were exposed to AgNP via water and food.

The ecological implications of a higher BAF for nanoparticles in comparison to the dissolved form of Ag may include the transfer of nanoparticles within a trophic chain that includes Daphnia. Since there is no evidence of internal translocation of nanoparticles inside the tissues of Daphnids (Lovern, Owen, & Klaper, 2008; Mendonça et al., 2011), a trophic transfer in this case will depend on the mechanisms of uptake and elimination of particles from the body that will ultimately rule their persistence inside the organism. According to our results, it can be suggested that AgNP will persist longer in the Daphnids body rather than ionic Ag.

4.5 Conclusions

In summary we observed that there were differences between AgNP and $AgNO_3$ patterns of bioaccumulation in *Daphnia magna* regarding their routes of exposure. By considering the worse-case scenario, wherein Ag is provided through water and food as sources of contamination and which happens to be the most expectable environmental situation, AgNP induced a higher bioaccumulation factor (BAF) in *Daphnia magna*. Unlikely single routes of exposure, the simultaneous presence of AgNP and algae created the most harmful conditions for Daphnia, ultimately leading to a higher bioaccumulation of AgNP when compared to the same exposure conditions to $AgNO_3$. We believe that these results can add valuable information for the undergoing construction of a proper and conservative assessment of nanoparticles effects in aquatic species, since the majority of studies performed so far related AgNP effects to Ag free ions. This is undoubtedly true for the majority of acute toxicity mechanisms, but it has been proved not to happen under chronic exposure scenarios and at environmental relevant circumstances, such as the presence of food in the media.

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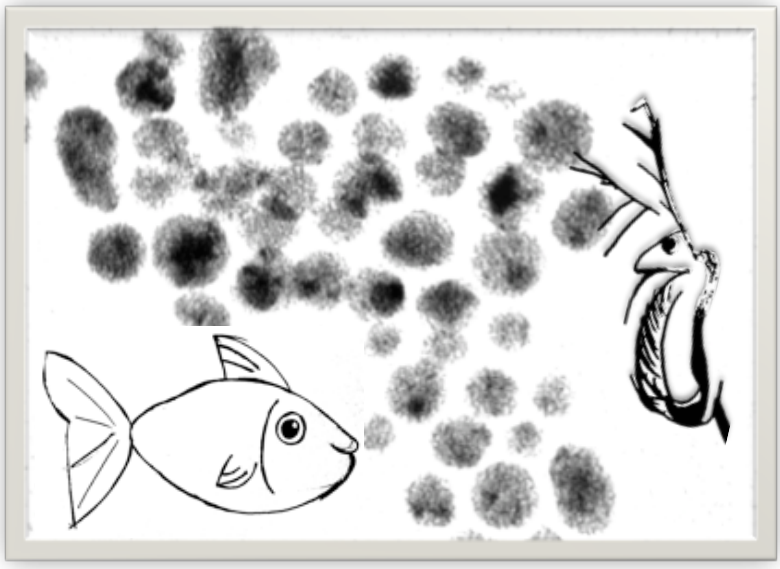
Chapter 5

Bioaccumulation of silver nanoparticles by *Carassius auratus:* a trophic transfer approach



5 Bioaccumulation and toxicokinetics of silver nanoparticles in the goldfish *Carassius auratus*: A trophic transfer approach

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Abstract

The increased use of silver nanoparticles (AgNP) as antimicrobial agent in consumer products and hospital utilities has raised concern regarding their waste products fate and their behavior in the environment. AgNPs are known to behave differently from Ag ions in the aquatic environment, and induce different toxic effects on aquatic species. These effects are, however, mostly reported on the basis of short term, acute exposures. Bioaccumulation, on the other hand, is a parameter that can enable the effect assessment of a contaminant even when it is present in the environment at a low concentration, which from an acute point of view would not cause any damage to an organism. The lower levels of an aquatic trophic chain can concentrate metals, and depending on their biological interactions and persistence, they can be magnified or just transferred along the trophic chain, and reach the higher levels. This process is well studied for regular metals but little is known about the mechanisms underlying metal based nanoparticles such as AgNP transfer within different species of an aquatic trophic chain. Therefore, the aim of this study was to evaluate the bioaccumulation pattern of AgNP in fish using a model aquatic chain composed by a green algae *Raphidocelis subcapitata*, a zooplankton species, *Daphnia magna*, and a freshwater fish *Carassius auratus* and compare it with the bioaccumulation of Ag ions (supplied by $AgNO_3$). In order to achieve that, fish were exposed to both water and contaminated food for an uptake period of 10 days followed by 7 days of elimination period. Fish was sampled at different time

points during uptake and elimination for Ag measurements in their different organs.

Our results suggest that the bioaccumulation of AgNP differ from $AgNO_3$. As a general pattern, the Ag concentration was higher in the organs of fish exposed to AgNP. For both treatments, the liver was the organ with lower Ag elimination rate, and no clearance of Ag from the liver was observed up to 7 days of elimination. Moreover, the Ag higher concentration in the muscle of fish was found upon AgNP exposure, suggesting that AgNP may have a higher chance of being transferred from lower to higher trophic levels.

5.1 Introduction

Silver nanoparticles (AgNP) are incorporated into a variety of products ranging from personal care products, food packing and medicine utilities, where AgNP is applied as an antimicrobial agent (Sotiriou & Pratsinis, 2010). Despite the recent exponential growth on nanomaterials production and application, which have raised concerns to the scientific and regulatory communities, nanosilver has been used, (although not with the prefix "nano"), since the late 1800s in the photograph industry and it has been registered under the U.S Environment Protection Agency (EPA) since 1970 to be applied as algaecide in swimming pools (Nowack, Krug, & Height, 2011). The worldwide estimative production of AgNP is of 250 to 312 tons per year (Hendren, Mesnard, Dröge, & Wiesner, 2011) from which is still unknown how much silver will end up in the environment. However, the environmental concentrations of Ag can be predicted by models that include data on Ag production, manufacturing of Ag-containing products, the silver ion release rate from products among others. Based on these models, Gottschalk et al. (Gottschalk, Sonderer, Scholz, & Nowack, 2009) reported that the Predicted Environmental Concentration (PEC) of silver in European surface waters was 0.76 ng/l, while Blaser et al. (Blaser, Scheringer, MacLeod, & Hungerbühler, 2008) predicted that in an intermediate scenario, Ag concentration in sewage treatment plant in Europe would be of 9 μg/L and in river waters, 140 ng/L. Although it is well recognized that AgNP will be released into the environment at any point during manufacturing, transport and use, there is still no specific regulation for the safe production and use of AgNP (or

other nanoparticles). The European Chemicals Agency responsible by the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) is undergoing a decision on whether specific regulation should be built for the use of nanomaterials or if the existing laws for regular chemicals are enough accurate and adapted to embrace nanoparticles (Pronk et al., 2009). This decision undoubtedly needs data from toxicity effects and potential for bioaccumulation of AgNP in aquatic and terrestrial organisms.

Many studies available nowadays on the effects of silver and silver nanoparticles focus on aquatic species to evaluate their potential risk.. These species are selected according to their relevance to the environment and the feasibility of data reproduction among laboratories. Based on these parameters, there are already important findings reported in the literature on the effects of AgNPs to aquatic species. Some examples are the study by Oukarroum et al. (Oukarroum, Bras, Perreault, & Popovic, 2012) on the effects of AgNP in a green algae *Chlorella vulgaris*, where it was observed that AgNP exposure to 1 mg/L induced the aggregation of algae cells, impaired their growth, reduced chlorophyll content and induced the formation of reactive oxygen species (ROS) inside the cells. The pores across the algae cell wall range in thickness from 5 to 20nm (Navarro et al., 2008) therefore is unlikely that AgNP will be internalized by algae. Instead, the observed effects are related to the concentration of particles agglomerates close to the cell surface, which can cause shading effects (Aruoja, Dubourguier, Kasemets, & Kahru, 2009) and the internalization of Ag^{+} released from AgNP (Ma, Geiser-Lee, Deng, & Kolmakov, 2010; Van Hoecke, De Schamphelaere, Van der Meeren, Lucas, & Janssen, 2008). Low AgNP concentrations were found to exert effects on life parameters of *Daphnia magna* such as feeding activity and

reproduction (Ribeiro et al., 2014; Zhao & Wang, 2011). For fish, AgNP was found to induce mortality and reduce hatching rates of *Danio rerio* embryos (Asharani, Lian Wu, Gong, & Valiyaveettil, 2008), induce developmental abnormalities such as absence of air sac, development of moderate and severe pericardial and yolk sac edema in fathead minnow embryos (*Pimephales promelas*) (Laban, Nies, Turco, Bickham, & Sepúlveda, 2009). In addition to all different effects that AgNP may induce in different species, the mechanism of toxicity of AgNP is rather dissimilar in some cases than the effects of the ionic silver (Ag^+). AgNP can be internalized in the organism where bigger Ag particles are not able to, and in some cases it may remain small enough to enter cells or cross the blood-brain-barrier (Shaw & Handy, 2011). Moreover and due to their size-specific characteristics, the persistence of NPs into biological systems may also differ from regular metals. If nanoparticles are more persistent in an organism, i.e., if its elimination rate is lower compared to a regular metal, than a higher magnification of nanoparticles is expected to occur inside a trophic chain. The majority of ecotoxicological studies on NP effects in aquatic animals are focused on short-term experiments with relatively high effect concentrations as a result. Bioaccumulation is considered a more conservative endpoint because it relies on the bioavailable chemical fraction, independent of the total dose that is available or provided. This fraction may represent a concentration that is not considered as harmful to the species, but yet has the potential to be uptaken and generate a body burden. Given that, bioaccumulation is an important parameter to be considered in environmental risk assessment for nanoparticles in order not to underestimate the risk they represent to the aquatic and terrestrial biota. In this study we aimed at measuring and studying the bioaccumulation and

possible Ag transfer to fish in a model aquatic trophic chain composed by *Raphidocelis subcapitata*, an unicellular freshwater algae, *Daphnia magna*, a zooplankton species with wide distribution and a model species in ecotoxicology, and *Carassius auratus*, the common goldfish, a successful invasive species in European waters and that can be used as a model fish species. In order to reproduce a worse case scenario of exposure, *C. auratus* was exposed to AgNP in water and fed with *Daphnia magna*, previously exposed through waterborne and dietborne AgNP. Silver nitrate ($AgNO_3$) was used in this study as an ionic control.

5.2 Methods

Organisms

Raphidocelis subcapitata cultures were maintained under laboratory conditions of constant light, temperature (20ºC± 1ºC) and aeration, in 10L gallons of Woods Hole MBL culture media, which had a known composition of salts and essential elements for algae growth. Initial concentration of algae cells was placed weekly in a gallon with freshly prepared culture media, and allowed to growth throughout 7 days, after which they reach a plateau phase of growth. At this stage, algae were centrifuged and supplied to *Daphnia magna* as food.

Daphnia magna, clone K6, originally from Belgium was also maintained and reproduced under laboratory conditions. Daphnia was cultured in ASTM media of moderated hardness and supplied with *R. subcapitata* as food, and seaweed extract as a source of micronutrients.

Carassius auratus were purchased from a local pet shop for the purpose of this study.

Fish used in this study were 2 months old, without sexual differentiation, averaging a 6 cm (± 0.5cm) size and weight 7g (± 1.7g). *C. auratus* were acclimated for 10 days in 20L aquariums containing tap water (pH 7.5, T 20ºC ± 1ºC) and fed with artificial food for the first days. During acclimation period, 10 fish were kept in each 20L aquarium, and trained to eat live food (*D. magna*). The water was renewed every 24h.

Chemicals

Silver nitrate was purchased from Sigma-Aldrich as a crystalline powder, 99% purity CAS 7761-88-8. Silver nanoparticles, with an alkane coating and with a small size range (3–8 nm) were supplied by AMEPOX (Poland). AgNP were provided as a suspension in water with initial concentration of 1000mg/L.

Nanoparticle characterization

Silver nanoparticles initial suspension (1000mg/L) was characterized in tap water by Transmission Electron Microscopy (TEM) technique. A sample of water was mixed with the AgNP stock dispersion at 100:1 volume ratio. A drop of the dispersion was deposited on a holey carbon coated Cu TEM grid and examined in a JEOL 2010 analytical TEM which has a resolution of 0.19 nm, an electron probe size down to 0.5 nm and a maximum specimen tilt of ±10° along both axes. The instrument is equipped with an Oxford Instruments LZ5 windowless energy

dispersive X-ray spectrometer (EDS) controlled by INCA.

Trophic transfer experimental design

In the present study we aimed at simulating a model aquatic trophic chain, constituted by *R. subcapitata*, *D. magna* and *C. auratus*, which were exposed to AgNP and $AgNO_3$ separately, through a worse-case scenario that could possibly occur for each of the species. In order to assess the transfer of Ag, either as ions or nanoparticles from algae to fish, algae was previously dosed for 6h with 1µg/L of AgNP and $AgNO_3$. The time of exposure was chosen based on previous bioconcentration experiments performed with *R. subcapitata*, in which it was observed that a maximum internal concentration of Ag was reached after 6h of exposure (Chapter 3). *Daphnia magna* was exposed for 24h (Chapter 4) through water containing the same concentration of Ag as the algae exposure (1µg/L). This concentration of 1µg/L was selected for both algae and daphnia based on some of the Predicted Environmental Concentrations (PEC) for silver. In addition, Ag pre-dosed algae were supplied as food to *Daphnia magna* during an AgNP contaminated water exposure. The exposure through water and dietary Ag reproduces the worse case scenario of Ag contamination that Daphnia could face in natural environments (Chapter 4).

Finally, *C. auratus* was dosed with 10 µg/L of AgNP and 5 µg/L of $AgNO_3$ through water exposure and fed daily with 2% wet body weight of *Daphnia magna* previously dosed with AgNP or $AgNO_3$. The concentration of water exposure of AgNP for fish was chosen based on the LOEC from acute exposure of the Japanese rice fish *(Oryzias latipes)* to AgNP, reported by Chae et al (2009). From

previous studies on the toxicity of AgNP and $AgNO_3$ for fish it is generally accepted that $AgNO_3$ exert higher toxicity than AgNP (Asharani et al., 2008; Ribeiro et al., 2014); therefore, half of the Ag mass of the AgNP exposure was applied as the ionic control ($AgNO_3$) as the concentration in the water for *C. auratus* exposure.

Feeding was taking place at the same time every day. In order to make sure that each fish ingested an equal amount of food, they were fed individually in 1l glass jars containing 20-25 adult Daphnia each. The experimental design was constituted of 3 replicates aquariums for AgNP and for $AgNO_3$. Water was daily renewed, after feeding, to avoid losses of Ag concentration in the media. Uptake phase was conducted for 10 days, after which the remaining fish in each aquarium were transferred to "clean" (Ag free) water and fed with non-contaminated Daphnia for a further period of 7 days, considered as the elimination phase. During uptake phase, one fish from each aquarium was collected at days 1, 3, 5, 7 and 10 and during elimination phase, fish were sampled at days 12, 14 and 17. After sampling, fish were sacrificed on ice by decapitation. Fish were not anesthized before decapitation since biomarker analysis such as acetilcolinesterase were performed in the remaining fish tissues (data not shown). All procedures involving fish handling were conducted according to the Guide for the Care and Use of Laboratory Animals of the European Union - in Portugal represented by Decreto de Lei nº 129/92 de 06 de Julho, Portaria nº1005/92 de 23 de Outubro de 1992.

The internal organs were separated for analyzes of their Ag content. Gill, liver, intestine and muscle from each fish were dried to a constant weight at 50ºC, then weighted and digested for Ag measurements.

Chemical digestion of water and tissue

Samples from fish water exposure were taken at time zero and after 24h of exposure. 10ml of water were collected in a 15mL falcon tube and afterwards digested for the analysis of silver concentration. 24h prior the digestion, all samples were mixed with HCl and H_2O_2 in order to bring their concentration in the sample to 1% and 5% respectively. After 24h the water was transferred to Teflon beakers and allowed to evaporate on a hotplate at 60ºC without boiling, until 1.5ml of sample was remained. After this step, samples were cooled down at room temperature and 3:1 ml of HCl:HNO_3 were mixed in all beakers. Samples were then evaporated over to 1.5ml (without boiling). The remaining volume was diluted to 45mL with 1% HCl solution. Total Ag was measured by Graphite-Furnace Atomic Absorption Spectroscopy (GF-AAS).

Tissue samples from algae, daphnia and fish organs were digested according to a modified version of EPA 3052 protocol (EPA 3052). Dry tissues were weighted and immediately placed in Teflon beakers with 3 ml of HNO_3 and heated on a hotplate for 30min with the Teflon's lids on. After 30 min of heating (or until the tissue was completely dissolved), 1 ml of HCl was mixed in the beakers and those were heated for another 30min with lids on. Afterwards the lids were removed and the acids were allowed to evaporate without boiling to a final volume of 1.5 ml. In addition, 3 replicates of the reference material DOLT-4 (Dogfish Liver Certified Reference Material for Trace Metals) were digested by the same method as the other tissue samples. The remaining content of each sample was

diluted with 1% HCl solution to a final volume of 45 ml, and analyzed by GF-AAS for their total silver content.

Kinetics modeling

The contribution of both water and dietary exposures to the accumulation of Ag in fish organs was modeled by the one compartment kinetics model, which describes the chemical fate using balance equations. We considered the various fish organs, each one as one compartment in which Ag kinetics will be studied. The concentration of Ag in the tissue will depend on the concentration of Ag in water and on the diet. The bioaccumulation of Ag in fish was therefore calculated by the balance concerning uptake (through water and food) and elimination, according to the equation:

$$Q(t) = \frac{k1w \times cw + k1f \times cf}{k2} \times (1 - e^{-k2 \times t)}$$

In which:

Q(t) – Concentration in the tissue at time t

k_1w – Uptake rate constant from water (L/g/day)

cw – Concentration in the water (µg/L)

k_1f –Uptake rate constant from food ($g_{daphnia}/g_{tissue}$/hour)

Cf – Concentration in the food (µg Ag/g daphnia dry weight)

k_2 – Elimination rate constant

The bioaccumulation Factor (BAF), estimated for each of the fish organs, was calculated as the ratio between the maximum concentration of Ag in the organ and the Ag concentration in the water.

$$BAF\ (organ) = \frac{C\ tissue}{C\ water}$$

The Biomagnification Factor (BMF) was calculated, again for of the fish each organ separately, as the ratio between the Ag concentration in the organ tissue and the Ag concentration in the fish diet. Biomagnification was assumed to occur if the BAF value of fish was higher then the BAF value of its diet (*Daphnia magna*).

$$BMF = \frac{C\ \text{tissue}}{C\ Daphnia}$$

5.3 Results

Nanoparticle characterization in tap water

Figure 5.1 shows the silver nanoparticles dispersed in the fish test-media (tap water). Ag particles dispersed in tap water showed possibly a different size distribution from the specified by the manufacture, with a number of very small particles present (1-2nm). That is likely to be related with the reaction products of dissolved Ag and ions in the tap water. The majority of particles however was within the 3-8nm range (fig. 5.1A and 5.1B). After 24h there was no substantial

difference compared to the fresh AgNP suspension, but possibly a lower number of the water reaction products.

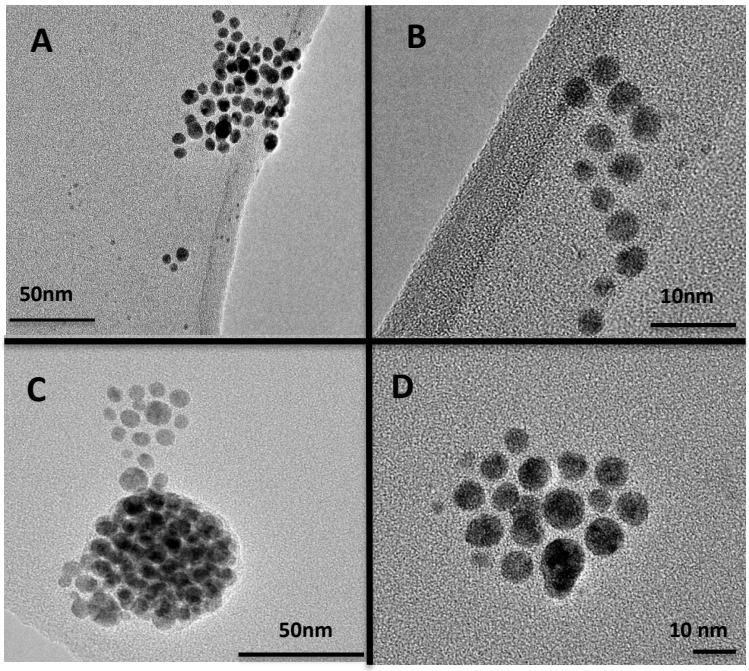

Figure 5.1. Silver nanoparticles TEM image in tap water (from Aveiro, Portugal). A and B represent the particles at time zero of exposure and figures C and D represent particles after 24h of exposure.

Analytical measurements

The recovery of Ag in all digested water samples was within a 90% range; therefore concentrations of silver are presented as nominal concentrations.

The concentration of Ag in *Daphnia magna,* which was supplied as food for *C. auratus* during the nanoparticle exposure was of 7.1 µg Ag /g/dw (SE=1.18) and for the silver nitrate exposure of 15.4 µg Ag /g/dw (SE=0.17). The concentration of Ag in the algae in which Daphnia was fed was 2.45 µg Ag /g/dw (SE=0.12) for AgNP and 4.04 µg Ag /g/dw (SE=1.68) for $AgNO_3$.

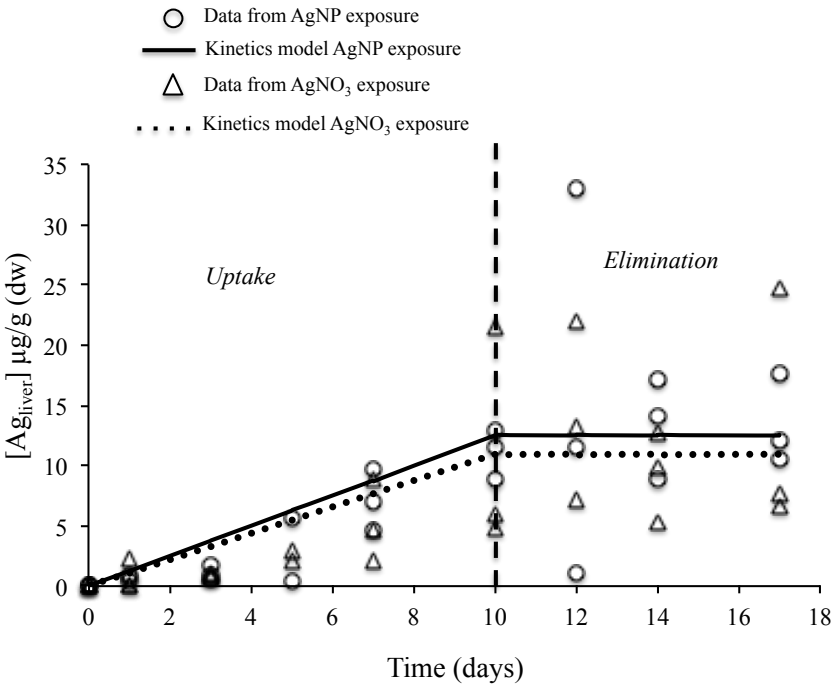

Figure 5.2. Kinetics of uptake and elimination of AgNP and $AgNO_3$ in the liver of *C. auratus*. The open circles represent the measured concentration in the liver and the lines stands for the kinetics model prediction. At day 10 fish were transferred to clean water for the Ag elimination period.

Toxicokinetics of Ag in Carassius auratus

The evaluation of the Ag kinetics in the different fish organs revealed distinctive accumulation patterns and therefore different parameters of uptake and elimination. In addition, a different pattern of accumulation of Ag was also found in fish depending on the Ag source of exposure (AgNP or $AgNO_3$). Fish exposed to AgNP showed higher Ag body burdens in the intestine, followed by liver, muscle and gills. On the other hand, fish exposed to $AgNO_3$ presented a higher concentration of Ag in the liver, followed by the intestine, gill and muscle.

Considering the kinetics of Ag in the intestine of *C. auratus*, we could observe a rapid Ag uptake already in the first days of exposure, in which fish exposed to AgNP reached higher concentrations compared to the $AgNO_3$ treatment.

There was no clearly decreased in Ag concentrations in the intestine of fish even after the 7 days of elimination phase, and this patterns showed to be also present in the liver tissues (Fig. 5.3). Regarding the model parameters for the intestine, K_{water} and K_{food} were lower for the $AgNO_3$ exposure compared to AgNP, while the elimination rate constant (k_2) was lower at AgNP exposure. The maximum average body burden found in the intestine of *C. auratus* exposed to AgNP was 30.10 (SE=12.54) µg/g (dw), reached at 2 days of elimination phase, while for $AgNO_3$, the average maximum concentration was 6.46 (SE=3.91) µg/g (dw), also achieved after 2 days of elimination (day 12).

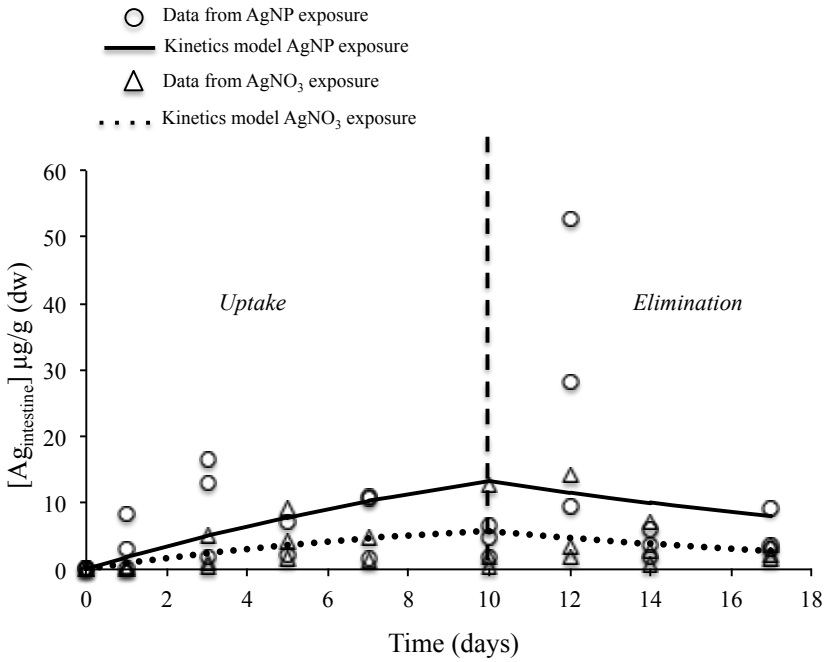

Figure 5.3. Kinetics of uptake and elimination of AgNP and $AgNO_3$ in the intestine of *C. auratus*. The open circles represent the measured Ag concentration in the intestine and the lines stands for the kinetics model prediction. At day 10 fish were transferred to clean water for the Ag elimination period.

By interpretation of the toxicokinetics parameters estimated by the model in the liver of *C. auratus*, the uptake rate constant from water (k_{water}) was exactly the same between AgNP and $AgNO_3$ exposures, and the uptake rate constant from food were very similar (table 5.1), whereas the elimination rate constant (k_2) was far lower in the liver of fish exposed to AgNP. Indeed, as represented in figure 5.2, Ag concentration in the liver remains very high even at 7 days post-exposure to AgNP and $AgNO_3$. On both exposure treatments, fish achieved a similar average maximum concentration in the liver, which was 15.26 (9.40) µg/g (dw) for AgNP, achieved after 2 days in the elimination phase, and 14.19 (4.33) µg/g (dw) for $AgNO_3$, also reached after 2 days fish were in the elimination phase (day 12).

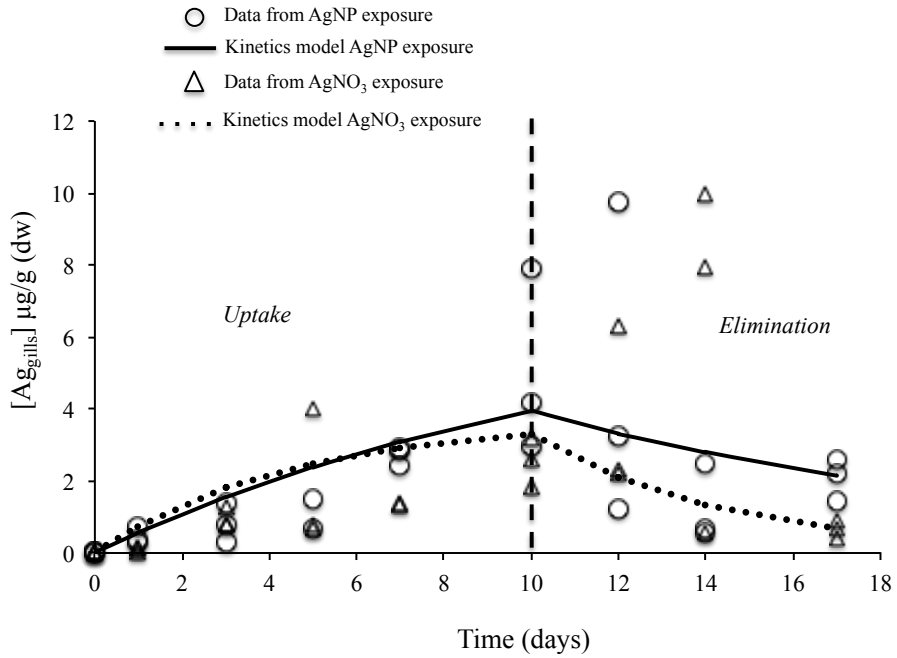

Figure 5.4. Kinetics of uptake and elimination of AgNP and $AgNO_3$ in the gills of *C. auratus*. The open circles represent the measured concentration in the gills and the lines stands for the kinetics model prediction. At day 10 fish were transferred to clean water for the Ag elimination period.

The gills of *C. auratus* were the only organ to show major differences between AgNP and $AgNO_3$ regarding the parameters values estimated by the kinetics model (Table 5.1). Higher k_{water} and k_2 values were estimated for the $AgNO_3$ exposure, while k_{food} was higher at the AgNP treatment. Moreover, the gill of fish exposed to AgNP achieved a maximum Ag concentration at day 10 of the uptake phase, which was 5.0 (SE=1.49) µg/g (dw), whereas in fish exposed to $AgNO_3$, Ag concentration in the gills at day 10 was 2.56 (SE=0.40) µg/g/dw. In addition, the higher Ag concentration in the gill of fish exposed to $AgNO_3$ was 6.17 (SE=2.87) µg/g/dw, which was achieved at the end of the elimination phase (day 14).

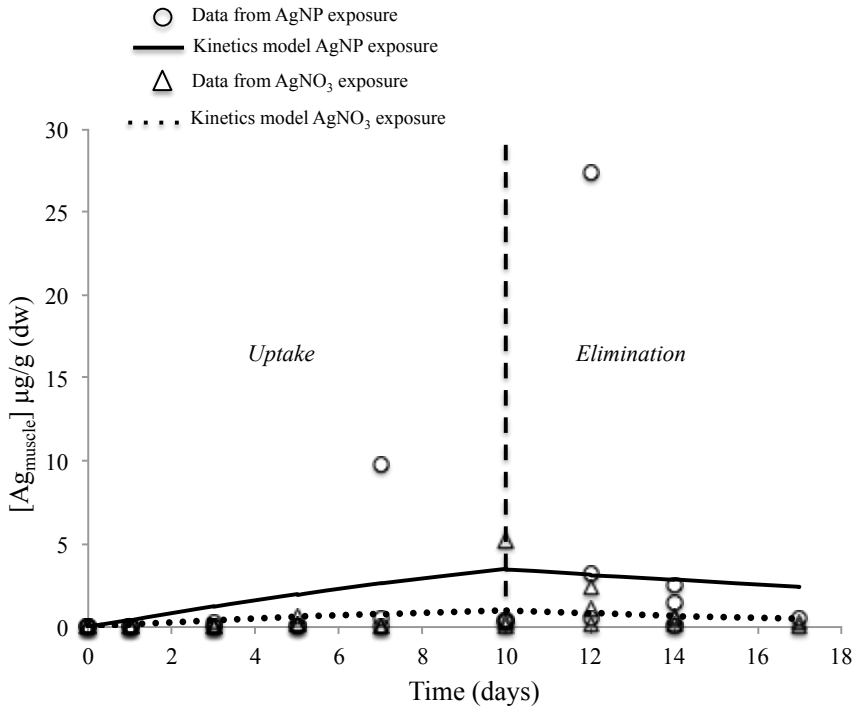

Figure 5.5. Kinetics of uptake and elimination of AgNP and $AgNO_3$ in the muscle of *C. auratus*. The open circles represent the measured concentration in the muscle and the lines stands for the kinetics model prediction. At day 10 fish were transferred to clean water for the Ag elimination period.

Considering the Ag concentration in the organs as a function of exposure time, the muscle was the ultimate organ where Ag concentrations were detected, upon the exposure of fish to $AgNO_3$, which was expected given the pattern of metal distribution in fish organs already reported elsewhere (see later discussion). Fish exposed to AgNP showed a maximum average concentration in the muscle greater than in the gills. The presence of Ag in the muscle tissue was detected after 3 days of exposure to AgNP and 5 days of exposure to $AgNO_3$.

The estimated parameters for the kinetics of Ag in muscle are also present in table 5.1. Those values shows that k_{water} was the same for fish exposed to both AgNP and $AgNO_3$, whereas k_{food} was higher at AgNP and k_2 lower, when compared to $AgNO_3$ exposure, indicating slower elimination rate upon AgNP exposure. Furthermore, the average maximum concentration of Ag found in the muscle of *C. auratus* was also reached 2 days after the elimination period started, with values of 10.40 (SE=8.53) µg/g (dw) for AgNP and 2.94 (SE=0.67) µg/g (dw) for $AgNO_3$ exposures.

Table 5.1. Parameters estimated by the kinetic model for the exposure of *Carassius auratus* to AgNP and $AgNO_3$ via Ag-contaminated water and food. The kinetics were calculated for each fish organ. K_{water} is the constant of uptake rate from water (L/g/hour), K_{food} is the constant of uptake rate from food (g/g/hour), K_2 is the elimination rate constant, C_{food} is the concentration of Ag in the food (daphnia) (µg/g dw), C_{water} is the concentration of Ag in the water (µg/L) and SS are the residues from the observed and predicted values.

	Organ	K_{water}	K_{food}	k_2	C_{food}	C_{water}	SS
AgNP	*Gill*	0.03	0.04	0.09	7.1	10	157
	Liver	0.07	0.07	0.0001	7.1	10	652.9
	Intestine	0.12	0.09	0.07	7.1	10	2764
	Muscle	0.01	0.05	0.05	7.1	10	722.3
$AgNO_3$	*Gill*	0.13	0.001	0.23	15.4	6.5	155.9
	Liver	0.07	0.06	0.03	15.4	6.5	717.7
	Intestine	0.08	0.03	0.10	15.4	6.5	256.2
	Muscle	0.01	0.003	0.11	15.4	6.5	6.15

5.4 Discussion

Among a few studies available in the literature dealing with the effects of nanoparticles in fish, this work aimed at investigating the patterns of distribution of silver nanoparticles (AgNP) in comparison to ionic silver (here provided as $AgNO_3$) in the various organs of *Carassius auratus* under simultaneously waterborne and diet Ag exposures. A one-compartment kinetics model was applied to study the kinetics of Ag in each of the fish organs. Our results suggest that the pattern of bioaccumulation of Ag differs between AgNP and $AgNO_3$ exposures. The Ag accumulation in fish exposed to $AgNO_3$ follows the

pattern of distribution among organs that was already described for other metals, including silver (Yamazaki, Tanizaki, & Shimokawa, 1996), with higher concentrations of Ag being found in the liver, followed by the intestine, gill and muscle. On the other hand, fish exposed to AgNP presented a different pattern of distribution, where the greater amount of Ag was found in the intestine, followed by liver, muscle and gills.

The exposure time of 10 days was not enough for fish to reach a plateau in Ag internal concentration neither on AgNP nor on $AgNO_3$ exposures. Therefore there was no equilibrium between the uptake and elimination of Ag during this period. Likewise, the 7 days of elimination phase were not sufficient to completely remove Ag from the organs (see later discussion).

By analyzing the kinetics parameters estimated by the model we could observe different values for uptake constants from water and from food (k_{water} and k_{food}) and elimination rate constants (k_2) concerning different target organs. This was somehow expected given that the different organs of fish have distinct affinity for metal uptake (Jezierska & Witeska, 2006). But one pattern that was similar in some of the cases was the lower Ag elimination rate from AgNP exposure when compared to $AgNO_3$. Our findings regarding Ag distribution in fish exposed to $AgNO_3$ are in accordance with some of the studies already reported in the literature. Yamazaki et al (1996) measured among other metals, the Ag levels in *C. auratus*, collected from the Asaka River in Japan, and found a similar pattern of concentration of Ag in the organs, being the liver the compartment with the higher Ag concentration, followed by the gallbladder, kidney and ovary. In this study, the intestine of fish was the organ with the second higher Ag concentration, and that is probable due the fluid that is segregated by the

gallbladder (which was proved to accumulate Ag) (Yamazaki et al., 1996) into the intestine to facilitate the fat metabolism (Govoni, Boehlert, & Watanabe, 1986) and therefore, a transfer of silver from the gallbladder to the intestine may have occurred in this case. Moreover, the silver concentration in the liver of *C. auratus* remained constant after the uptake period of both AgNP and $AgNO_3$ and did not decrease even after 7 days of exposure to uncontaminated water, indicating a failure of Ag excretion by the liver. By the interpretation of the model parameter of elimination constant (k_2) in the liver, those submitted to AgNP had an elimination rate constant much lower than the liver of fish exposed to $AgNO_3$. Nonetheless, although there is no clear evidence of Ag elimination from the liver (Fig. 5.2), we can assume, based on the estimated k_2 values, that if fish would have enough time to eliminate silver, this process would take longer to occur on those fish exposed to AgNP through water and food exposures, indicating an higher risk to aquatic systems

From a literature review, it is unanimous that the liver is the major organ for accumulation of metals in fish (Scown et al., 2010; Shaw & Handy, 2011; Wood, Playle, & Hogstrand, 1999; Yamazaki et al., 1996), and that the levels of accumulated metals may remain high in the liver for a long period of depuration (Jezierska & Witeska, 2006). However, little is known about the mechanisms of uptake and effects of AgNP into the liver cells and tissue that could be associated with a smaller elimination rate when compared with Ag ions. In addition, exposure through water was two times higher for AgNP than for AgNO3, and it was exactly the opposite when regarding food exposure, where daphids exposed to AgNO3 showed twice the body burden than those exposed to the particulate form. Therefore it is also difficult to extrapolate from exposure levels. In a study

by Choi et al. (2010), histological alterations in the liver of zebrafish were observed upon exposure to AgNP (Choi et al., 2010). Those alterations included disruption of hepatic cell cords and apoptotic changes as chromatin condensation. From a toxicogenomic point of view, Gagné et al. (Gagné et al., 2012) suggested that the mechanisms of toxicity of Ag^+ ions and AgNP in the liver of rainbow trout are dissimilar: while Ag^+ ions involves the mobilization of metals and oxidative stress, the AgNP is related to other pathways including inflammation and cell protein denaturation (Gagné et al., 2012). In addition, it has been proved that exposure of fish to Ag^+ (Hogstrand, Galvez, & Wood, 1996) and AgNP (Chae et al., 2009) increased the levels of metallothionein (MT) in the fish liver. Moreover, in the study of Chae et al (2009), there was a significant higher induction of MT on the *Japanese* medaka (*Oryzias latipes*) exposed to AgNP when compared to $AgNO_3$. As MTs are related to the immobilisation of metals such as cadmium and copper inside the cells by means of binding to the toxic metals thus decreasing its the toxicity by decreasing its bioavailability, (Amiard, Amiard-Triquet, Barka, & Pellerin, 2006; Din & Frazier, 1985), it can be assumed that one of the mechanisms behind the lower elimination of Ag from the liver of goldfish in this study is related to the induction of MT, consequently to the immobilisation of Ag in the cells.

The kinetics of Ag in the intestine of *C. auratus* was relatively different between exposures to AgNP and $AgNO_3$. When exposed to AgNP via water and food, fish uptake higher amounts of Ag compared to $AgNO_3$. The kinetic model parameters of Ag for the intestine also suggest that under AgNP exposure, uptake rates from water and food were higher and the elimination rate was lower compared to $AgNO_3$. Interestingly, the higher concentration of Ag in the intestine of fish was

observed at day 2 of the elimination phase in the AgNP exposure. In this study, *C. auratus* was fed with *Daphnia magna* t previously exposed to Ag-contaminated water and food (algae). Since *C. auratus* lacks a stomach, the highest amount of Ag found in the intestine may be attributed to its diet (Daphnia previously contaminated with AgNP). According to the results obtained in chapter 4, Daphnia exposed to AgNP through water and food has the tendency to store AgNP for longer period of time, when compared to ionic Ag (chapter 4). Therefore, it is assumed that the higher amounts of AgNP in the intestine of fish may be a reflection of its dietary content. Nonetheless, at the end of the elimination phase, Ag levels in the intestine were still higher compared to the control group, which highlights the finding that elimination of Ag by the intestine is slower for AgNPs.

Copper and silver share similar chemical characteristics, especially regarding their interaction with cell membranes. According to Clearwater et al (2000), the entrance of copper in the intestinal epithelium of fish is a passive process occurring through the basolateral membrane (Clearwater, Baskin, Wood, & McDonald, 2000), which may also apply for silver uptake mechanism. The fish gills are considered a negatively charged ligand to which positively charged metals in solution will compete for binding sites (Hogstrand & Wood, 2009). Measured concentrations of Ag in the gills of fish exposed to AgNP were the same between the last day of exposure (day 10) and the 2 first days of elimination (day 12), indicating a possible presence of AgNP agglomerates into/onto the gills that could not been eliminated. In an in vitro study with gill cells of rainbow trout, Farkas et al (2011) reported that clusters of AgNP were visualized inside the gill cell of rainbow trout after 48h of exposure to citrate-capped AgNP. The

agglomerates were found around the nuclei and no evidence of AgNP was seen within the nuclei. Moreover, no effective clearance of AgNP from the cells was observed after 48h of extensive washing and transfer of cells to NP-free medium. Based on these findings, there is a strong evidence that the measured Ag in the gills of *C. auratus* during elimination phase in this study is related to previously absorbed AgNP into/onto the gills. Regarding $AgNO_3$ exposures, the higher level of Ag in the gills was measured at the 4^{th} day of elimination. Ag^+ is known to have greater bioavailability than AgNP, which makes Ag^+ ions to be easily transported between different compartments of an organism due to its ability to be taken up by cells (Campbel, 1995). In this study it was observed that the higher levels of Ag were found in the liver and intestine of *C. auratus* and that elimination of Ag from the liver was nearly zero for 7 days post-exposure to AgNP and $AgNO_3$. Because the liver has a central location in the circulatory system of fish and lack a basement membrane, the toxicant exchange between the blood and the hepatocytes is maximized in fish liver (Joo, Kalbassi, Je Yu, Lee, & Johari, 2013). In the light of these evidence, it can be assumed that the higher amounts of Ag found in the gills of fish during elimination period was attributed to the circulatory system, in which the Ag present in the liver was possibly transported to the gills. The white muscles are known to have less blood circulation, thus being the last organ where toxicants are found in the fish body. In this study the white muscles of fish exposed to $AgNO_3$ contained the lower levels of Ag among all other organs. Ag concentration in the muscle of *C. auratus* exposed to AgNP was five-fold higher than the $AgNO_3$ and reached a peak on the 2^{nd} day of elimination period. This peak is related to the higher AgNP concentration found in other organs of fish such as gills, intestine and liver on the 2^{nd} day of

elimination. The presence of a toxic substance in the white muscle tissue of fish is usually associated with a failure of the liver on the detoxification activity (Jebali, Banni, Gerbej, & Boussetta, 2008). Although the MT levels in the liver were not measured in this study, it is expected that enzymatic activity of the liver played a role in the presence of AgNP in the muscle.

Nonetheless, as the liver is the major organ involved on fish metabolism, and also plays a part on detoxification process, our results suggests that a higher retention rate (indicated by small k_2) of AgNP in the liver may cause the transference of Ag to the muscle tissue, which is the main tissue of concern for human health purposes (Agah, Leermakers, Elskens, Fatemi, & Baeyens, 2008). Long term consequences of this process are also likely to occur, and those may involve dissolution of the retained particles inside the liver, membrane damage and earlier cell apoptosis, and most important, the potential of trophic transfer of AgNP to predatory fish, birds or mammals.

5.5 Conclusions

The bioaccumulation profile of AgNP in the goldfish *Carassius auratus* differ from ionic Ag, when different organs were analyzed separately. Upon $AgNO_3$ exposure to both Ag- contaminated water and food, the highest concentration of Ag was fond in the fish liver, while for the AgNP exposure, the intestine was the organ with higher levels of Ag in *C. auratus*. For both exposure treatments, the liver was the organ with the lower elimination rate constant (k_2), and no decrease on Ag concentration was observed neither for AgNP nor for $AgNO_3$ exposure up to 7 days of the elimination period. Fish exposed to AgNP also demonstrated a higher gill concentration during the elimination period, which also suggested to be associated with the binding of Ag particles to the gills or to be internalized in the cells. In conclusion, this study provided indications that bioaccumulation is an essential parameter to be taken into the risk assessment of metal based nanomaterials, since nanoparticles have distinct types of interactions with biological membranes and tissues, and should not be dealt as a regular metal contaminant. Therefore, we strongly recommend specific regulations for the complete and reliable assessment of the risk that nanoparticles may induce on the environment.

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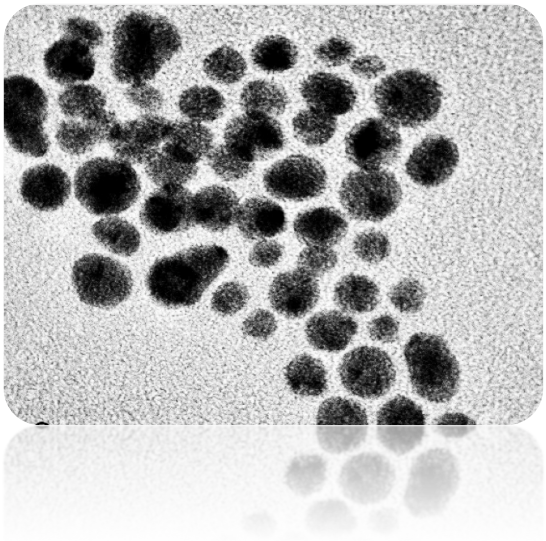
Chapter 6

Summary & Final conclusions



6 Summary and final discussion

This PhD thesis aimed at studying the biological aspects guiding the interactions of silver nanoparticles (AgNP) with three model freshwater species that have ecological relevance and which play an important role in aquatic food chains. The objectives and questions that guided the conduction of this work were defined in the general introduction; the results are presented in the main body of the thesis within chapters 2 to 5, and in this section there will be provided a summary of the most important findings as well as some final considerations regarding the relevance of this work and future perspectives.

In **Chapter 2** the toxic effects of silver nanoparticles on life parameters of the three species used in this study are presented and compared with those effects of ionic silver (as $AgNO_3$) and discussed on the basis of Effective-Concentration (EC_{50}). This chapter represented an important step for the subsequent work presented here because it provided information on concentrations of AgNP that would be reliable for the assessment of bioaccumulation on the same species.
The concentration of silver nanoparticles inducing 50% of *R. subcapitata* growth inhibition was similar to ionic silver, although it was assumed that the mode of interaction of particles and ionic silver with the algae cells is dissimilar: while AgNP form agglomerates in the algae growth media, that may act as shading agents, interfering with photosynthesis and growth, ionic Ag may be internalized by the algae cells through the membrane channels (Chapter 3 provides a more detailed discussion on this). AgNPs were 10 times less toxic to *Daphnia magna*

than ionic Ag, and the presence of food decreased the toxicity for both AgNP and ionic Ag, however the results indicate that the presence of food (algae) in the acute test with *Daphnia magna* exhibit a higher "protective" effect on the nanoparticle exposure, verified by the 6-fold increase in the LC_{50} of AgNP versus a 3-fold increase in the LC_{50} value for $AgNO_3$. This was again, reasonable related to the type of interaction between nanoparticles and algae cells; it is known that algae segregates exopolymeric substances (EPS) that can induce the agglomeration of particles, decreasing its bioavailability to Daphnia, consequently its toxicity. Furthermore, silver nanoparticles were less toxic to *Daphnia magna* when compared to silver ions, in all other parameters analyzed in chapter 1. Finally in this chapter the effects of AgNP were evaluated on the embryo development of Zebrafish (*Danio rerio*) and compared with the effects induced by ionic Ag. On the basis of effective-concentration, ionic silver was more toxic to *Danio rerio* embryos, with a lower 48h EC_{50} value for mortality. However it is to emphasize that although AgNP caused lower mortality rate on zebrafish, it induced different embryo abnormalities than $AgNO_3$, such as the presence of pericardial edema, which was observed only under AgNP exposure.

Danio rerio was chosen over *Carassius auratus* for the evaluation of the impacts of AgNP in the fish embryo development due to several reasons: the eggs of *D. rerio* are transparent, which allows the observation in time of their embryonic development and the detection of any abnormalities induced by the toxicant; there are existing studies dealing with the assessment of AgNP effects in *Danio rerio* embryo development, which allowed the comparison of our results with literature data. Moreover, the FET (fish embryo toxicity) is a standard protocol and it has proven to be a reliable alternative method for the regular acute

toxicity tests with juvenile fish, avoiding the need for a large volume of test solution, and the use of juvenile fish, reducing vertebrate testing as requested by the European Union.

Having characterized the toxicity of AgNP to the aquatic model species, the bioaccumulation studies started with the green algae *Raphidocelis subcapitata*, where, in **chapter 3,** it was presented a research regarding the influence of silver nanoparticles behavior in solution and speciation of the released ionic Ag from AgNP on the bioconcentration of AgNP and ionic Ag by this algae species. It was verified that bigger sized particles have less influence on the bioconcentration of Ag by algae, which was proved by the kinetics parameters calculated for nanoparticle exposure. The small sized (dissolved and ionic Ag) induced higher Bioconcentration Factor (BCF) in algae, suggesting that dissolved Ag species and ionic Ag are probably concentrated by algae. Moreover, the use of the CARS microscopy technique revealed that this algae species does not internalize these silver nanoparticles.

On **chapter 4**, the bioaccumulation of AgNP by *Daphnia magna* was studied under different exposure routes: water, food and water + food. It was observed that when Daphnia was exposed to AgNP via water and dietary routes, the Bioaccumulation Factor (BAF) was higher compared to all other routes of exposure. In this case, nanoparticles were mainly uptaken via water, given the fact that algae do not internalize AgNP; therefore diet did not play a major role on bioaccumulation by Daphnia under joint exposure to water and food. However, food does interfere with AgNP behavior in solution, as mentioned above. Therefore, the probability of Daphnia to ingest agglomerated particles was increased. While the uptake of particles as agglomerates has different

mechanism than the uptake of soluble complexes, the elimination of these particles is also a mechanic activity that may be difficult to process, leading to a low predicted elimination constant.

Finally, in **chapter 5,** a full assessment of the trophic transfer of silver nanoparticles within a model trophic chain was conducted, using the goldfish *Carassius auratus* as the higher trophic level species. The uptake phase of 10 days in which *C. auratus* was exposed to both AgNP contaminated water and food (*Daphnia magna*) was not sufficient to induce equilibrium in the internal Ag concentration in fish. Neither 7 days of elimination period was enough for *C. auratus* to clear the previously ingested Ag from their organism. The higher concentration of AgNP was found in the intestine of fish, followed by the liver, muscles and gills. The liver presented the lower elimination constant compared to all other organs, suggesting that AgNP have a significant persistence in this organ.

Within the final aim of this thesis, reproducing a trophic chain scenario that includes all three model species that were studied previously provided some insights on the possibility of trophic transfer of AgNP. Fish exposed to AgNP through water and food (previously internalized with AgNP) was found to accumulate silver in a dissimilar way regarding fish different organs, where some organs concentrated higher amounts of Ag than others. In a food chain perspective this information is important, considering the proportion in which the tissues constitute the animal and what is the body part of the fish that will be eaten by a top predator.

For the purpose of estimating biomagnification of AgNP within the chosen model aquatic trophic chain, it was attempted on calculating the Biomagnification

Factors (BMF) from algae to Daphnia, and from Daphnia to fish, under two exposure scenarios: using concentrations of exposure applied in the chapter 3 and 4 (algae and Daphnia bioaccumulation studies) and using the concentrations applied in chapter 5, which were an attempt to reproduce a more likely environmental condition, in which algae and daphnia were exposed to 1 µg/L of AgNP. Therefore, the BMF from algae to Daphnia was calculated as follow:

$$BMF\ (Algae - Daphnia) = \frac{CDaphnia}{Calgae} = \frac{20.51}{27.71} = \mathbf{0.74}$$

The units of $C_{Daphnia}$ and C_{alage} are in µg/g (dw). The values were derived from the exposure scenario in which Daphnia was exposed to AgNP contaminated algae and uncontaminated water.

Afterwards, the biomagnification factor Daphnia-fish was calculated by using the AgNP concentration in each of the fish organs:

Gills: $BMF\ (Daphnia - Fish) = \frac{Cgills}{Cdaphnia} = \frac{5}{20.51} = \mathbf{0.24}$

Liver: $BMF\ (Daphnia - Fish) = \frac{Cliver}{Cdaphnia} = \frac{15.26}{20.51} = \mathbf{0.74}$

Intestine: $BMF\ (Daphnia - Fish) = \frac{Cintestine}{Cdaphnia} = \frac{30.10}{20.51} = \mathbf{1.46}$

Muscle: $BMF\ (Daphnia - Fish) = \frac{Cmuscle}{Cdaphnia} = \frac{10.40}{20.51} = \mathbf{0.50}$

The values representing concentrations in the organs are equivalent to the maximum AgNP concentration achieved in that organ during exposure period, and are reported as µg/g (dw).

Biomagnification is considered to occur only if the bioaccumulation factor (BAF) from a lower level is increased, or magnified in the next trophic level (see chapter 1 for definitions of bioaccumulation and biomagnification). Accordingly, in this study, the BMF calculated in the **intestine** of fish suggested that biomagnification of AgNP occurred for this organ, which is indicated by an

increase in the value BMF daphnia-fish$_{intestine}$ compared with the BMF algae-daphnia: 1.46 vs. 0.74, respectively.

In the second approach for the biomagnification estimation, the concentration in the algae and daphnia were derived from the exposure performed in chapter 5 in where both species were dosed with 1µg/L of AgNP before being provided as food for fish. Because in this experimental design, Daphnia was exposed to both AgNP-contaminated water and food, the total exposure concentration for Daphnia was transformed in order to obtain a final AgNP concentration in µg/L (See chapter 3 for detailed description on these calculations). Therefore, the BMF algae-daphnia was calculated as:

$$BMF\ (Algae - Daphnia) = \frac{CDaphnia}{Calgae + Cwater} = \frac{7.01}{2.91} = \mathbf{2.40}$$

By using 2.40 as a BMF for comparison value, the follow step was to verify if the any of the BMF (Daphnia-fish) organs will be higher than this value:

Gills: $BMF\ (Daphnia - Fish) = \frac{Cgills}{Cdaphnia} = \frac{5}{7.01} = \mathbf{0.71}$

Liver: $BMF\ (Daphnia - Fish) = \frac{Cliver}{Cdaphnia} = \frac{15.26}{7.01} = \mathbf{2.17}$

Intestine: $BMF\ (Daphnia - Fish) = \frac{Cintestine}{Cdaphnia} = \frac{30.10}{7.01} = \mathbf{4.29}$

Muscle: $BMF\ (Daphnia - Fish) = \frac{Cmuscle}{Cdaphnia} = \frac{10.40}{7.01} = \mathbf{1.48}$

In agreement with the first approach, biomagnification was only observed in the **intestine** of goldfish, suggested by the increase in the BMF daphnia-fish value when compared with the value previously obtained for algae-daphnia. On the other hand, if we consider the BMF algae-daphnia obtained in the first approach, where daphnia was exposed to AgNP via food only, then it is assumable that biomagnification can occur for every fish organ. However with the purpose of

extrapolation of these results into relevant environmental conditions, it is safer to cogitate that biomagnification of AgNP among the species used in this thesis, will probable occur only in the intestine of goldfish. For an assumption of ecological implications of this finding, one has to comprise the conditions in which the predation mechanisms happen. For instance, if a fish is eaten by a predatory fish or a bird, the biggest nutritional benefit from the fish prey to the fish or bird predator relies on the prey muscle tissue, due to its bigger proportion in the prey body, compared to the other organs. Nonetheless, the persistence of AgNP in a prey's organ (in this case, goldfish), even in a small tissue proportion on the body, may represent a hypothetical source of AgNP intake by a possible predator, potentially leading to unknown biological effects, depending on probability of nanoparticles to remain or not as particles after the physiological process of digestion and detoxification.

Risk characterization approach

Knowing the potential of a substance for bioaccumulation is a valuable step on environmental risk assessment because it supports the identification of those substances that may impact biological receptors, and can affect organisms at higher trophic levels, including humans (Munns, 1998). Considering the current state of nanoparticles risk assessment for the environmental compartment, data on bioaccumulation is of great value to assist an accurate risk assessment and characterization of NPs. Given that, the data acquired in this thesis can represent a source of information on risk characterization.

In **chapter 2** it was reported a study on the toxicity of silver nanoparticles to three different aquatic species; three species that are worldwide used as model species in ecotoxicology, and which also represents three different levels of an aquatic trophic chain. Short-term acute and sub-lethal assays were performed using several endpoints of those species (Ribeiro et al., 2014). In order to characterize the risk posed by silver nanoparticles to aquatic species, the lowest Non-Observed-Effect-Concentration (NOEC) from all endpoints were collected from chapter 2. Therefore, the lowest NOEC obtained for AgNP, among the three species was equivalent to 0.5 µg Ag/L, which was the highest concentration that had no effect on the reproduction of *Daphnia magna*. For the calculation of the Predicted Non Effect concentration (PNEC), a safety factor of 50 was used, considering that the NOEC was obtained among three different species and acute and chronic tests. Safety factors of 10 or 50 are advised if chronic testing data are available and are dependent on the extent of such data (Chapman & Fairbrother, 1998). In that way, the PNEC was obtained the lowest NOEC divided by a safety factor of 50, which equals to 0.01 µg/L.

The Predicted Environmental Concentrations (PEC) of silver nanoparticles for European surface waters were driven from three reports from Blaser et al (2008), Gottschalk et al (2009) and from the public deliverable 6.3 (NanoFATE, D.6.3) (https://wiki.ceh.ac.uk/display/nanofate/Home), in which the concentration of AgNP in the environmental was predicted by models that takes into account the amount of manufactured products which have incorporated AgNP, their persistence in the environment and the Ag release rate from the product. According to the public deliverable 6.3 from the NanoFATE FP7 EU

project, the highest concentration of AgNP that aquatic animals will be exposed to over a 11 years' period can reach up to 6ng/L in the European Union.

In the study by Blaser et al (2008), Ag predictions of Ag concentrations found in the environment were calculated (modelled) as silver released from textiles and plastics containing silver nanoparticles. However the prediction for environmental concentrations in this study not only includes AgNP, but also Ag in many environmental forms such as sulfates, chlorides and silver ions. Three different scenarios of surface water concentrations of Ag were predicted for Europe in this study. In these scenarios the levels of Ag were considered as minimum, intermediate and maximum. The concentrations of Ag predicted for each of the scenarios were, in **ng/L**, as follow: Minimum scenario – 40; Intermediate scenario – 140; Maximum scenario – 320.

Given that, it is possible to calculate a Risk Quotient ($\mathbf{R_Q}$) for silver based on those three reports. The present risk characterization is based on the Technical Guidance Document (TGD), part III of the EU (ECB, 2003). Considering the PNEC obtained in this study (0.01 µg/L), and the different PEC in which the calculations will be based on, the environmental risk of Ag for freshwater species can be calculated as the ratio between the PEC and the PNEC. Based on the public deliverable D.6.3 from the NanoFATE project, the environmental risk of silver nanoparticles for freshwater species in Europe will be:

$$R_Q = \frac{6\ (ng.L^{-1})}{10\ (ng.L^{-1})} = 0.60\ (R_Q<1)$$

According to the R_Q value, based on the PEC from the D.6.3, there is no immediate risk caused by the presence of AgNP at this concentration for freshwater species

in European surface waters. But the similarity of both PEC and PNEC values cannot be disregarded.

In addition, the R_Q was estimated by using the PEC reported for each of the scenarios predicted in the study by Blaser et al (2008), Therefore, for each of the scenarios, the risk estimation will be:

Minimum: $R_Q = \frac{40\ (ng.L^{-1})}{10\ (ng.L^{-1})} = 4$ (R_Q>1)

Intermediate: $R_Q = \frac{140\ (ng.L^{-1})}{10\ (ng.L^{-1})} = 14$ (R_Q >1)

Maximum: $R_Q = \frac{320\ (ng.L^{-1})}{10\ (ng.L^{-1})} = 32$ (R_Q >1)

As the results demonstrate, the risk coefficient is higher than 1 for all the scenarios, which means that for those PEC values there is environmental risk regarding silver. Considering the fact that those predictions were created in 2008 for what would be the situation in 2015 concerning the presence of Ag in the environment as a contribution from the products' release, one should recognize that our current situation is potentially harmful for aquatic species and can even become worse.

On the other hand, in the study by Gottschalk et al (2009), PEC is reported on the basis of Engineered Nanomaterials (ENM) only. Therefore, the output on this study predicted concentrations for several nanomaterials in different compartments of the environment, including AgNP in European surface waters. According to these findings, the concentration of AgNP in surface waters would be of 0.764 ng/L. Therefore, the risk quotient for AgNP calculated on the basis of the NOEC presented on chapter 2 and using the PEC from the study of Gottschalk et al (2009), will be:

$$R_Q = \frac{0.764\ (ng.L^{-1})}{10\ (ng.L^{-1})} = 0.0764\ \ (R_Q<1)$$

If a safety factor of 1000 is applied, the R_Q increases to 1.52, which is similar to the R_Q obtained in their study (1.1). However, it is not clear whether the values for the PNEC calculation are from one single species and endpoint, or if the values refer to acute and/or chronic studies. Nevertheless, a risk quotient higher than 1 for AgNP, by using a factor of 1000 does not necessarily mean that there is risk for aquatic species, but it emphasize the need for further investigations to gather a valuable database of effects on aquatic species, in order to support the calculation of PNEC´s which will benefit the risk characterization of nanoparticles in the environment.

Additionally, the ecotoxicological data regarding freshwater species that is presented in this thesis can represent an additional source of information for the Species Sensitivity Distribution (SSD) approach concerning silver nanoparticles, which is based on the NOEC from several species (Aldenberg & Jaworska, 2000) (Postuma et al, 2002). Likewise, the SSD method support the calculation of a 5% Hazard Concentration (HC5), a parameter that represents the substance concentration causing effects on 5% of the species allocated in the SSD. The HC5 in turn, is usually compared with the PNEC for risk characterization methods.

Conclusion and future perspective

Currently, nanotechnology is still in exponential growth. Although there is a great scientific effort on recognizing the potential harm that nanomaterials can represent to the environment, there are still gaps to be filled in concerning their risk as nanomaterials. In addition studies like those presented can help also

industry to design their nanomaterials in a safer way, in order to reduce risk (Safe by Design).

The 4-years study presented in this thesis validates important aspects regarding nanoparticle toxicity for model aquatic species in Europe: from adaptation of bench methodologies to the analysis of potential transfer of silver nanoparticles within a model trophic chain. It is evidenced that ionic Ag is more toxic to the model species. However, when analyzing bioaccumulation potentials, toxicity effects of ionic Ag seems not to be relevant, given that silver nanoparticles were found to accumulate more in our model species than ionic Ag. With this, it expected that biological mode of action of nanoparticles must be considered when assessing their effects on the environment. Finally, the use of a safety factor of 50 for calculating the risk quotient for nanoparticles, based on currently available PEC´s, revealed that risk for aquatic species can be a reality and more studies on nanoparticles fate and modeling predicting should also be carried out.

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